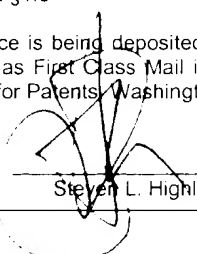


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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Paul McCray *et al.*

Serial No.: 09/448,613

Filed: November 22, 1999

For: METHODS AND COMPOSITIONS FOR
INCREASING THE INFECTIVITY OF
GENE TRANSFER VECTORS

Group Art Unit: 1641

Examiner: R. Schnizer

Atty. Dkt. No.: IOWA:022/SLH

APPEAL BRIEF

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APPEAL BRIEF

BOX AF
Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

This Appeal Brief is filed in response to the Final Office Action mailed on July 18, 2002,. The two-month date for submission of the Appeal Brief was on December 22, 2002, by virtue of the date (October 22, 2002) stamped on the return postcard filed with the Notice of Appeal filed on October 15, 2002. The deadline for filing of this brief is February 22, 2003, by virtue of the enclosed Petition for Extension of Time.

A request for a two-month extension of time to submit the Appeal Brief is included herewith along with the required fee. In the event that the request and necessary fee is deficient or absent, the Commissioner is authorized to deduct the fees for this brief from Fulbright & Jaworski L.L.P. Account No. 50-1212/IOWA:022US/SLH. Please date stamp and return the attached postcard as evidence of receipt.

I. Status of the Claims

Claims 1-70 were filed with the application. Claims 9, 40 and 45 have been canceled. Claims 13-25, 57-59, 61 and 62 have been withdrawn from consideration. Thus, claims 1-8, 10-12, 26-39, 41-44, 46-56, 60 and 63-70 are under examination, are pending in the application, and stand appealed. A copy of the appealed claims is included herein as Appendix A.

II. Status of the Amendments

No amendments have been offered after the Final Office Action dated July 18, 2002.

III. Statement of Interest

The real party in interest is the assignee, the University of Iowa Research Foundation.

IV. Related Appeals and Interferences

There are no related appeals or interferences.

V. Summary of the Invention

The invention is directed to a method for increasing the susceptibility of epithelial cells to viral infection by increasing transepithelial permeability with a tissue permabilizing agent. The epithelial cells may of any epithelial tissue type but, in particular embodiments is airway epithelial tissue, most particularly airway epithelial tissue selected from the group of tracheal, bronchial, bronchiolar and alveolar tissue. Specification, page 4, lines 9-14; lines 23-26.

In another embodiment the susceptibility of epithelial cells to viral infection by increasing the transepithelial permeability may be further modified by increasing the

proliferation of the epithelial cells by contacting them with a proliferative factor. Any proliferative factor may be used, but in a particular embodiment the proliferative factor is a growth factor. In further embodiments, the proliferative factor may be delivered as an aerosol or as a topical solution. Specification at page 4, lines 16-21.

The tissue permeabilizing agent may be selected from a group including hypotonic solutions, ion chelators, cationic peptides, occludin peptides, peptides designed to disrupt extracellular portions of the junctional complexes, cytoskeletal disruption agents, antibodies, ether, neurotransmitters, glycerol, FCCP, oxidants, and mediators of inflammation. In further specific embodiments, the ion chelator may be EGTA, BAPTA or EDTA; the cationic peptide may be poly-L-lysine; the cytoskeletal disruption agent may be cytochalasin B or colchicine; the neurotransmitter may be capsianoside; the oxidant may be hydrogen peroxide or ozone; and the mediator of inflammation may be $\text{TNF}\alpha$. The antibody may be an anti-E-cadherin antibody. Specification at page 4, line 26 to page 5, line 4.

Yet another embodiment provides a method of increasing the susceptibility of epithelial cells to viral infection by increasing the transepithelial permeability via the paracellular route, further comprising infecting the epithelial tissue with a virus vector selected from the group including virus from the virus families retrovirus, adenovirus, parvovirus, papovavirus and paramyxovirus, from the virus genera lentivirus and adeno-associated virus, and the vaccinia virus. This embodiment is further modified in still further embodiments wherein the viral vector contains a non-viral gene under the control of a promoter active in eukaryotic cells. Any non-viral gene may be used, but in a particular embodiment the non-viral gene is a human gene, and in yet another embodiment the human gene encodes a polypeptide selected from the group consisting of a tumor suppressor, a cytokine, an enzyme, a toxin, a growth factor, a membrane

channel, an inducer of apoptosis, a transcription factor, a hormone and a single chain antibody. In another embodiment the virus vector may be a replication-defective virus, and in a further embodiment the replication-defective virus is a retroviral vector. Specification at page 5, lines 8-21.

In still another embodiment there is provided a method of increasing the susceptibility of epithelial cells to viral infection by increasing the transepithelial permeability wherein the epithelial tissue is diseased. In a further embodiment the disease of the epithelial tissue may be lung cancer, tracheal cancer, asthma, surfactant protein B deficiency, alpha-1-antitrypsin deficiency or cystic fibrosis. Specification at page 5, lines 23-27.

As a further embodiment, the invention provides a composition comprising both a tissue permeabilizing agent and a cell proliferative factor suitable for aerosol application, and in another embodiment, suitable for topical application. Any tissue permeabilizing agent may be used in either composition, but in a further embodiment the tissue permeabilizing agent of the composition is selected from the group of a hypotonic solution, a cytokine, a cationic peptide, a cytoskeletal disruptor, a mediator of inflammation, an oxidant, a neurotransmitter or an ion chelator. It is understood that any proliferative factor can be used in the aforementioned compositions. An additional embodiment of the compositions further comprises a packaged viral vector. The packaged viral vector in other embodiments comprises a non-viral gene or is a retroviral vector or other gene transfer vector. Specification at page 5, line 29 to page 6, line 8.

The invention also provides a method for redistributing the viral receptors or enhancing accessibility of viral receptors on epithelial cells of an epithelial tissue by increasing the transepithelial permeability of the epithelial tissue. This may involve opening the paracellular pathway and allowing vectors free access to receptors on the basolateral membrane. Any viral

receptor may be redistributed, but in another embodiment the viral receptor is a retroviral receptor. Specification at page 6, lines 10-15.

The invention provides a further embodiment which is a method for expressing a polypeptide in cells of an epithelial tissue comprising the steps of (a) providing a packaged viral vector comprising a polynucleotide encoding said polypeptide; (b) increasing the permeability of said epithelial tissue; and (c) contacting cells of the epithelial tissue with the packaged viral vector under conditions permitting the uptake of the packaged viral vector by the cells and expression of said polypeptide therein. Other embodiments of this method further comprises increasing the proliferation of cells in the epithelial tissue or further comprises a viral vector which is a retroviral vector. Specification at page 6, lines 16-24.

Also, the invention provides a method for treating epithelial tissue disease comprising the steps of (a) providing a packaged viral vector comprising a polynucleotide encoding the therapeutic polypeptide; (b) increasing the permeability of the diseased epithelial tissue; and (c) contacting cells of the epithelial tissue with the packaged viral vector under conditions permitting the uptake of the packaged viral vector by the cells and expression of the therapeutic polypeptide therein, whereby expression of the therapeutic polypeptide treats the disease. A further embodiment of the method comprises increasing the proliferation of the cells of the diseased epithelial tissue. Any means of increasing the proliferation of the cells of the diseased epithelial tissue may be used but a further embodiment the means of increasing the proliferation is contacting the epithelial cells with a proliferative agent. Another further embodiment of the method comprises a method in which the diseased epithelial tissue being treated is airway tissue. Any airway tissue may be treated, but a further embodiment treats airway tissue selected from the group of alveolar tissue, bronchiolar tissue, bronchial tissue and

tracheal tissue. A further embodiment of the method is one which comprises the treatment of epithelial tissue disease wherein the disease is cancer. Any cancer may be treated but further embodiments are directed to the treatment of lung cancer or tracheal cancer. Specification at page 6, line 26 to page 7, line 11.

In still another embodiment of the method, the epithelial tissue disease being treated is an inherited genetic defect. The invention is directed to any inherited genetic defect, but further embodiments are specifically directed to the inherited genetic defects surfactant protein B deficiency, alpha-1-antitrypsin deficiency or cystic fibrosis. The method for treating an epithelial tissue disease has a further embodiment wherein the method further comprises the use of a therapeutic polypeptide selected from the group consisting of a tumor suppressor, a cytokine, an enzyme, a toxin, a growth factor, a membrane channel, an inducer of apoptosis, a transcription factor, a hormone and a single chain antibody. Another embodiment of the method for treating epithelial tissue disease is one in which the step of increasing the permeability of the diseased epithelial tissue comprises contacting cells of said diseased epithelial tissue with a tissue permeabilizing agent. Finally, the method of treating an epithelial tissue disease has an embodiment in which the viral vector used is specifically a retroviral vector. Specification at page 7, lines 13-25.

VI. Issues on Appeal

Are claims 1, 3, 4, 5, 7-12, 16-51, 53, 56-66 enabled under 35 U.S.C. §112, first paragraph?

Are claims 1, 2, 4, 6-8, 26-31 and 48-52 anticipated under 35 U.S.C. §102(b) by anticipated by Halbert *et al.* (Exhibit A)?

VII. Grouping of the Claims

The claims stand or fall together with respect to the enablement rejections under 35 U.S.C. §112, first paragraph.

The claims stand or fall together with respect to the anticipation rejections under 35 U.S.C. §102(b).

VIII. Summary of the Argument

Appellants acknowledge that cystic fibrosis disease pathogenesis remains controversial, as do approaches for new therapies, including gene transfer. However, the examiner has provided a slanted view of the prior art, focusing on the most negative aspects of gene therapy, and ignoring more favorable reviews. In addition, the action stands as an indictment of gene therapy generally, which applicants believe is not representative of the view of those of skill in the art. The examiner goes even further in creating an unreasonable standard for cystic fibrosis therapy, based on equating "therapy" with "cure."

Moreover, the present invention is a classic "improvement" invention, and it should be examined as such. Thus, *any* application this invention might have would enable its use, despite the narrow focus presented here in light of the election of species requirement. The election of species requirement imposed should thus have been applied as a vehicle to reduce the scope of the examiner's burden in searching prior art. Since no relevant prior art was found, the election of species requirement should have been withdrawn. Instead, the examiner has seen fit to

continue limiting the scope of review to treatment of cystic fibrosis – but only for the purpose of determining enablement – and at the same time examining a far broader reading of the claims for prior art purposes.¹

Turning to scientific issues of enablement, the examiner maintains that it is controversial which specific cell types must be transduced in order to correct the CF defect. However, there are significant data supporting the idea that correction of chloride transport defect is fundamental, and the present inventors' clearly show that it is possible to correct the chloride transport defect over significant periods of time, and through different routes. And though there is no clear indication how many cells must be transduced, and what level of expression must be attained, the data presented certainly demonstrate effective transfer and expression of genetic material. Thus, any ambiguity should be decided in the favor of the appellant, as the burden lies squarely with the PTO in this regard. Finally, the additional "concerns" the examiner might raise are ones that address therapies generally, and are not specific to the claims at issue.

Finally, with regard to the prior art rejection, it is sufficient to state that the rejection has no foundation. Halbert *et al.* teaches no "composition that comprises a tissue permeabilizing agent." It teaches use of an instrument to wound tissue, and certainly describes no therapy. Thus, in order to construe this reference as anticipating the claimed invention, the examiner has abandoned any logical meaning for these claim terms.

¹ The examiner admits that Halbert *et al.* disclose no therapies, but the §102(b) rejection is maintained.

IX. Argument

A. *Standard of Review*

As an initial matter, appellant notes that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an examiner's position on appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. *Rejection Under 35 U.S.C. §112, First Paragraph*

Claims 1-8, 10-12, 26-39, 31-44, 46-56, 60 and 63-70 stand rejected under 35 U.S.C. §112, first paragraph. Once again, however, appellants respectfully submit that the examiner's position is incorrect, both factually and legally, as explained below.

Background on Treating Cystic Fibrosis

At the outset, it is important to understand that several issues central to the pathogenesis and treatment of cystic fibrosis remain unresolved scientifically in the time since the CFTR gene was discovered in 1989. The disease pathogenesis remains controversial, as do approaches for new therapies, including gene transfer. However, appellants believe the examiner has provided a

slanted view of the prior art, focusing on the most negative aspects of gene therapy, and ignoring more favorable reviews. In addition, the action stands as an indictment of gene therapy generally, which applicants believe is not representative of the view of those of skill in the art, *e.g.*, Crystal (1999) (Exhibit B).

In rebuttal, the examiner argues initially that Crystal (1999) is not relevant as “published after the filing date” of the instant application. While Crystal (1999) was indeed published after the filing date of the instant application – by about a week – it is more than a bit disingenuous to argue that its content does not reflect the views of those skilled in the art *at about the time the application was filed*. Crystal (1999) is a review and, as such, reflects a cumulative knowledge of those in the field, as evidenced by the fact that 18 of the 23 references cited by Crystal are dated 1998 and before. Thus, it is entirely improper for the examiner to dismiss the significance of Crystal (1999).

The Examiner has Set Up an Improper Standard for Review

Before delving into the scientific merits of the rejection, appellants would like to point out that the entire framework for the rejection is improper. The presently claimed methods represent a significant advance in the application of viruses to gene transfer involving epithelia. In particular, the present specification contains irrefutable evidence, both *in vitro* and *in vivo*, that indicates the claimed invention provides a dramatic improvement in viral gene transfer into epithelial cells. The invention is a classic “improvement” invention, and it should be examined as such. Thus, any application this invention might have would enable its use.

Unfortunately, through an election of species requirement, the examiner has focused the inquiry on a single clinical situation – cystic fibrosis. While entirely proper from a PTO

procedure standpoint, the election of species severely distorts the enablement issue. If the *only* use for the claimed invention was in the treatment of cystic fibrosis, the scrutiny being applied might be appropriate. However, there are many other uses for the invention. The election of species requirement should thus have been applied as a vehicle to reduce the scope of the examiner's burden in searching prior art. Since no prior art was found regarding cystic fibrosis, the election of species requirement should have been withdrawn. Instead, the examiner has seen fit to continue limiting the scope of review to treatment of cystic fibrosis – but only for the purpose of determining enablement – and at the same time examining a far broader reading of the claims for prior art purposes.²

The examiner goes even further, however, in creating an unreasonable standard for cystic fibrosis therapy. As set forth in both office actions, the examiner argues at length about what percent of cells must be transduced in order to achieve “normal chloride ion transport *in vivo*.” This is tantamount to requiring “therapy” to equal “cure.” Yet even with the rigorous reading of the claims now posited by the examiner, nowhere has it been argued that a cure is required. The balance of the discuss regarding how much expression is required, and how this translates into a percentage of cells that must be transduced is academic. The bottom line is that appellants have demonstrated an increase in CFTR expression *in vivo*. The examiner then has a burden to *demonstrate* – not hypothesize – that even a minimal increase is of no value to the subject. In fact, the examiner *admits* that “the degree of correction needed for clinical benefit of these defects is unknown.” What better indication can one offer that the examiner has not been able to overcome the presumptively enabling nature of appellants’ disclosure? *In re Marzocchi*, 169 USPQ 370 (CCPA 1971).

² The examiner admits that Halbert *et al.* disclose no therapies, but the §102(b) rejection is maintained.

"The prior art teaches that it is not known which cells must be transfected with CFTR expression vectors in order to treat CF."

As pointed out in the first Office Action, it is generally true that it remains controversial which specific cell types must be transduced in order to correct the CF defect. However, there are significant data supporting the idea that correction of chloride transport defect is fundamental. Loss of cyclic AMP activated chloride conductance in airway epithelia is the fundamental physiologic hallmark of cystic fibrosis. The CFTR chloride channel has been studied extensively, and there is general consensus that one key function of this channel is to transport chloride. This function is defective in cystic fibrosis. From a prevailing perspective, it may not be necessary to know which cell types to transduce as long as the end result is restoration of chloride transport. This is the general strategy that is being taken by the field. What is required is restoration of CFTR function (chloride conductance), which appellants have achieved (below).

The present inventors *in vitro* data using primary cultures of airway epithelia derived from patients with cystic fibrosis clearly show that it is possible to correct the chloride transport defect in this model for 11 months' duration with retroviral vectors ("Figure A," previously submitted; extended version of Figure 2b, Wang *et al.*, 1999; Exhibit C). Based on the turnover of epithelial cells in this model, in which cells are slowly proliferating, these data suggest that the results stem from transduction of a population of cells with progenitor capacity that have gradually repopulated the epithelium. This duration of expression is unprecedented in the cystic fibrosis gene literature and speaks to the efficacy of the present invention and the ability of retroviral transgenes integrate and persist. These results also verify that a retroviral vector integrated into the host cell genome can persist in a population of progenitor cells in a manner

sufficient to correct the chloride transport defect. Given this ability, appellants submit that the instant specification is sufficiently enabling, even with the caveat discussed above regarding target cells. The examiner also focused initially on the potential of correcting the CFTR defect in submucosal gland epithelia. It is true that submucosal gland cells express CFTR in greater abundance than surface epithelia. This has led some investigators to speculate that the role of CFTR in the submucosal gland cells may be critical in disease pathogenesis. However, there is no universal agreement in this regard and the role of submucosal glands in disease pathogenesis continues to be debated. The first Office Action also argued that it is critically important that a vector be delivered by the systemic circulation in order to target the submucosal glands rather than deliver a vector topically through the airway lumen. Others have tried to deliver viral vectors systemically to target airway and submucosal gland epithelia, and the efficiency of this approach has been low. Lemarchand *et al.* (1994) (Exhibit D). Furthermore, there is evidence from studies in transgenic animals to suggest that precise delivery of the therapeutic gene to its normal cell type of expression may be required for therapeutic benefit in CF. For example, Zhou *et al.* (1994) (Exhibit E) showed that expressing CFTR in the intestinal surface epithelia using the FABP promoter corrected the fatal intestinal phenotype in CFTR-null mice despite the fact that the CFTR expression was directed to surface epithelia rather than the crypt epithelia in which it is normally expressed in high abundance. Appellants offered that this is not only the best evidence of cell-type non-specificity, it is the *only* evidence of record on this point. Thus, speculation of certain commentators notwithstanding, appellants believe that this study still argues persuasively against the examiner's position.

In response to this line of argument, the examiner's only argument is that "there is no evidence that the instant invention can be used to obtain the extent of gene expression found in a

transgenic animal.” The examiner misstates the both the question at hand, and the relevant legal test. First, the question at hand was whether appellants were focusing on the correct cell type. As stated, the only evidence of record is Zhou *et al.*, which clearly indicates that surface epithelia can provide a suitable environment for exogenous CFTR expression. Moreover, the examiner alludes to “expression throughout the life of the animal.” Again, appellants are not advancing a single dose cure for CF. Thus, even short term expression can provide clinical benefits to a patient.

Second, the relevant legal standard is whether there is evidence of record to **doubt** that one can achieve sufficient expression, not affirmative evidence to the contrary. This inappropriate standard is again echoed in the examiner’s closing statement that “it is not clear that Applicant’s invention could be used to treat CF by transfection of surface epithelium only.” Appellants emphasize, at the risk of being repetitive, that it is the examiner who has the burden here, and merely raising interesting biologic questions does not suffice to shift the burden.

“It is also unclear how many cells must be transfected and what level of gene expression is required to achieve therapy.”

While it is true that gene therapy for CF is not being routinely practiced at this time, and that there is no detailed protocol to which applicants can point, such a situation does not preclude enablement. There are *in vitro* studies showing that adding between 6% and 10% normal cells to a population of CF cells corrects the chloride transport defect. Johnson *et al.*, 1992 (Exhibit F). Thus, it is not the case that correction of the CFTR defect in 100% of cells would be required, although the precise number of cells required *in vivo* is not known. However, though such potential knowledge gaps may exist, this does not preclude a finding that the current invention is enabled. “We hold as we do because it is our firm conviction that one who has taught the public

that a compound exhibits some desirable pharmaceutical property in a standard experimental animal has made a significant and useful contribution to the art, even though it may eventually appear that the compound is without value in the treatment of humans.” *In re Krimmel*, 130 USPQ 215 (CCPA 1961).

The examiner’s response is to agree, but note that “gaps in the knowledge ... show that treatment of CF by gene therapy is extremely unpredictable.” Unpredictability is not the basis for a rejection under §112, first paragraph, however. It is one factor in considering whether an specification is enabling. Here, appellants have both *in vitro* and *in vivo* data, and there are volumes of work by those in the field that continue to pursue gene therapy of airway epithelia as a way of treating CF. Nothing more is required for enablement:

[The appellant] is arguing that there must be a rigorous correlation between pharmacological activity between the disclosed *in vitro* utility and an *in vivo* utility to establish practical utility. We, however, find ourselves in agreement with the Board that, based on the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence.

Cross v. Iizuka, 224 USPQ 739 (Fed. Cir. 1985) (finding sufficient §112 support for a priority claim), citing *Nelson v. Bowler*, 206 USPQ 881 (1980). Moreover, a recently published paper demonstrates that use of a lentiviral vector expressing CFTR can be transferred to the respiratory epithelium using a formulation that disrupts tight junctions (mild detergent). Limberis *et al.*, *Human Gene Ther.* 13:1961-70 (Nov. 1, 2002) (Exhibit G).³ In that report, correction of the ion transport defect was attained and could be measured for months.

³ As stated in MPEP §715.09 (Seasonable Presentation): “... [E]vidence traversing rejections [is] considered timely if submitted ... after final rejection and submitted ... with a satisfactory showing under ... 37 C.F.R. §1.195” 37 C.F.R. §1.195 in turn states that “exhibits submitted after the case has been appealed will not be admitted without a

Thus, there is every reason to believe that the biological activity shown by appellants can be utilized to improve what the field considers the most promising approach to CF therapy. Given the evidence provided, and the very nature of the invention, it is not reasonable to require appellants to provide rigorous proof of enablement for every disease state that could possibly be treated with the claimed invention.

“Boucher (1999) teaches that it is likely that the percentage of epithelial cells requiring functional correction to restore normal chloride ion transfer in vivo may well exceed 10%”

As stated above, the examiner is improperly considering only those treatment conditions that would “restore normal chloride ion transfer,” which is not the relevant consideration. In so doing, the examiner has placed considerable weight on the study of Boucher (1999) (Exhibit H), which reported that more than 10% of epithelia may be required to accomplish a therapeutic goal. This hypothesis is based largely on work at a single center, and is not accepted as the fundamental issue facing CF therapy. These studies used adenoviral vectors in a time before the receptor for the virus was recognized and, not surprisingly, the transfer efficiency was low. The present invention directly affects this problem by increasing the viral transduction of target cells. Appellants’ own publications, *in vivo* studies have achieved up to 14% transduction of tracheal epithelia, and up to 10% of epithelia in small airways. Wang *et al.* (1999) (Exhibit C); Wang *et al.* (2000) (Exhibit I). In an unpublished manuscript, transduction of rabbit tracheal epithelia exceeds 10% in some areas. Taken together with the showing that nasal perfusion of respiratory epithelia with an EGTA solution decreased transepithelial electrical potential, indicating opening of tight junctions, these papers indicate a tremendous potential for gene therapy in humans.

showing of good and sufficient reasons why they were not presented earlier.” The publication of the Limberis *et al.* paper after the filing of appellants’ Notice of Appeal would appear to constitute such good and sufficient reason.

The examiner's response to this well-supported argument is to state that "[t]his assertion is unsupported by evidence." Does the examiner deny that the reference use adenoviruses? Does the examiner deny that the study was done prior to discovery of the adenoviral receptor? The examiner also attacks Wang *et al.* (1999) as showing 14% transduction at best, but only an average of 4.8% +/- 5.6%. However, appellants submit that these number support enablement. The examiner also argues that Wang *et al.* (2000) fails to show 10% transduction anywhere. While the number "10%" is not stated, Figures 5D and 5E do indeed show that greater than 10% of the epithelia is transduced. Again, it should be emphasized that this report shows that EDTA can cause changes in the bioelectric properties of *human nasal epithelium in vivo*, without causing adverse symptoms.

"[T]he accumulation of mucus associated with the CF in humans ... impedes vector access to epithelium"

In the first Office Action, it was argued that mucus in patient airways presents yet another hurdle to the use of gene therapy. However, if the therapy is applied to infants and young children prior to the development of this symptom, the airways will not present the same barriers. As such, this fact cannot preclude a finding of enablement for the simple reason that it does not apply to patients across the board.

The examiner argues, in response, that appellants have failed to provide any evidence that infants and children do not have inhibitory amounts of mucous, and that it is the examiner's concern that mucous will present a significant challenge to gene therapy. Thus, it is the *examiner's* burden to establish that an unreasonable number of cystic fibrosis patients have an unreasonable amount of mucous. Appellants submit that the record is deficient on this point. It also is argued that appellants have failed to consider the effects of surface fluid and cilia on

transfection. However, recently published data demonstrate an *in vitro* model of differentiated human airway epithelia. Karp *et al.* (March, 2002); (Exhibit J)⁴. The cells are heavily ciliated, the cilia beat, and the cells secrete salt, water and mucus. As illustrated in this report, the methods of the present invention work well, *even taking into account surface fluids and cilia*. Thus, though not necessary, appellants can now point to additional evidence that refutes the examiner's concerns.

C. *Rejection Under 35 U.S.C. §102*

Claims 1, 2, 4, 6-8, 26-31 and 48-52 stand rejected under 35 U.S.C. §102(b) as anticipated by Halbert *et al.* The reference is said to teach increasing susceptibility to retroviral infection of rabbit tracheal cells *in vivo* by abrading (wounding). Appellants again traverse.

As submitted in the first response, appellants claims recite that the method by which permeability is increased is by application of *composition* that comprises a tissue permeabilizing agent. This clearly distinguishes the methods of Halbert, which administer no composition, but instead rely on mechanical abrasion or wounding of the tissue.

In the final Office Action, the examiner maintained this rejection, arguing that "the brush of Halbert can be considered the tissue permeabilizing agent." This argument misstates the issue. The issue is whether Halbert anticipates the claims. The present claims recite "treating said cells with composition that comprises a tissue permeabilizing agent." The question,

⁴ As stated in MPEP §715.09 (Seasonable Presentation): "... [E]vidence traversing rejections [is] considered timely if submitted ... after final rejection and submitted ... with a satisfactory showing under ... 37 C.F.R. §1.195" 37 C.F.R. §1.195 in turn states that "exhibits submitted after the case has been appealed will not be admitted without a showing of good and sufficient reasons why they were not presented earlier." As this is the first submission by appellants' since the examiner's clarification of the rejection the Final Office Action, this would appear to constitute such good and sufficient reason.

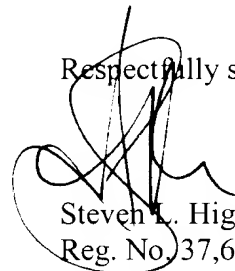
therefore, is whether Halbert "treats" a cell with "a composition" comprising "a tissue permeabilizing agent."

Appellants again submit that to interpret Halbert as using "a composition" is obtuse, to say the least. A brush is not a composition of matter, and it certainly is not a composition of matter that itself comprises a separate tissue permeabilizing agent. Thus, the examiner has absolutely no basis for arguing that Halbert teaches the same invention as appellants. As such, the rejection should be summarily reversed.

X. Conclusion

In light of the foregoing, appellants respectfully submit that the claims on appeal should not be rejected under 35 U.S.C. §112, first paragraph, on the basis of enablement. Reconsideration and withdrawal of the rejection is respectfully requested.

Respectfully submitted,



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Date: February 24, 2003

APPENDIX A: PENDING CLAIMS

1. A method for increasing the susceptibility of epithelial cells to viral infection comprising treating said cells with composition that comprises a tissue permeabilizing agent, whereby an increase in transepithelial permeability increases the susceptibility of said epithelial cells to viral infection.
2. The method of claim 1, wherein said epithelial tissue is airway epithelial tissue.
3. The method of claim 2, wherein said airway epithelial tissue is bronchial tissue.
4. The method of claim 2, wherein said airway epithelial tissue is tracheal tissue.
5. The method of claim 2, wherein said airway epithelial tissue is alveolar tissue.
6. The method of claim 1, further comprising increasing the proliferation of said epithelial cells.
7. The method of claim 6, wherein increasing the proliferation of said epithelial cells is achieved by contacting said cells with a proliferative factor.
8. The method of claim 7, wherein said proliferative factor is a growth factor.
10. The method of claim 9, wherein said tissue permeabilizing agent is a hypotonic solution.
11. The method of claim 9, wherein said tissue permeabilizing agent is ion chelator.
12. The method of claim 11, wherein said ion chelator is EGTA, BAPTA or EDTA.

13. (Withdrawn) The method of claim 9, wherein said tissue permeabilizing agent is a cationic peptide.
14. (Withdrawn) The method of claim 13, wherein said cationic peptide is poly-L-lysine.
15. (Withdrawn) The method of claim 9, wherein said tissue permeabilizing agent is an occludin peptide.
16. (Withdrawn) The method of claim 9, wherein said tissue permeabilizing agent is a cytoskeletal disruption agent.
17. (Withdrawn) The method of claim 16, wherein said cytoskeletal disruption agent is cytochalasin B or colchicine.
18. (Withdrawn) The method of claim 9, wherein said tissue permeabilizing agent is ether or glycerol.
19. (Withdrawn) The method of claim 9, wherein said tissue permeabilizing agent is a neurotransmitter.
20. (Withdrawn) The method of claim 19, wherein said neurotransmitter is capsianoside.
21. (Withdrawn) The method of claim 9, wherein said tissue permeabilizing agent is FCCP.
22. (Withdrawn) The method of claim 9, wherein said tissue permeabilizing agent is an oxidant.
23. (Withdrawn) The method of claim 22, wherein said oxidant is hydrogen peroxide or ozone.

24. (Withdrawn) The method of claim 9, wherein said tissue permeabilizing agent is a mediator of inflammation.
25. (Withdrawn) The method of claim 24, wherein said mediator of inflammation is $\text{TNF}\alpha$.
26. The method of claim 1, further comprising infecting said epithelial tissue with a virus vector selected from the group consisting of a retrovirus, a lentivirus, an adenovirus, an adeno-associated virus, a parvovirus, a papovavirus, paramyxovirus and a vaccinia virus.
27. The method of claim 26, wherein the virus vector comprises a non-viral gene under the control of a promoter active in eukaryotic cells.
28. The method of claim 27, wherein said non-viral gene is a human gene.
29. The method of claim 28, wherein said gene encodes a polypeptide selected from the group consisting of a tumor suppressor, a cytokine, an enzyme, a toxin, a growth factor, a membrane channel, an inducer of apoptosis, a transcription factor, a hormone and a single chain antibody.
30. The method of claim 26, wherein the virus vector is a replication-defective virus.
31. The method of claim 30, wherein the virus vector is a retroviral vector.
32. The method of claim 1, wherein said epithelial tissue is diseased.
33. The method of claim 32, wherein said disease is lung cancer, tracheal cancer, asthma, surfactant protein B deficiency, alpha-1-antitrypsin deficiency or cystic fibrosis.
34. The method of claim 7, wherein said proliferative factor is delivered as an aerosol.

35. The method of claim 7, wherein said proliferative factor is delivered as a topical solution.
36. The method of claim 9, wherein said tissue permeabilizing agent is delivered as an aerosol.
37. The method of claim 9, wherein said tissue permeabilizing agent is delivered as a topical solution.
38. A composition suitable for aerosol application comprising a tissue permeabilizing agent, a cell proliferative factor and a packaged viral vector.
39. The composition of claim 38, wherein said tissue permeabilizing agent is a hypotonic solution, a cytokine, a cationic peptide, a cytoskeletal disruptor, a mediator of inflammation, an oxidant, a neurotransmitter or an ion chelator.
41. The composition of claim 38, wherein said packaged viral vector comprises a non-viral gene.
42. The composition of claim 38, wherein said packaged viral vector is a retroviral vector.
43. A composition suitable for topical application comprising a tissue permeabilizing agent, a cell proliferative factor and a packaged viral vector.
44. The composition of claim 43, wherein said tissue permeabilizing agent is a hypotonic solution, a cytokine, a cationic peptide, a cytoskeletal disruptor, a mediator of inflammation, an oxidant, a neurotransmitter or an ion chelator.
46. The composition of claim 43, wherein said packaged viral vector comprises a non-viral gene.

47. The composition of claim 43, wherein said packaged viral vector is a retroviral vector.
48. A method for redistributing viral receptors on epithelial cells of an epithelial tissue comprising increasing the transepithelial permeability of said epithelial tissue, whereby increased transepithelial permeability facilitates redistribution of said viral receptors on said epithelial cells.
49. The method of claim 48, wherein said receptor is a retroviral receptor.
50. A method for expressing a polypeptide in cells of an epithelial tissue comprising:
- (a) providing a packaged viral vector comprising a polynucleotide encoding said polypeptide;
 - (b) increasing the permeability of said epithelial tissue; and
 - (c) contacting cells of said epithelial tissue with said packaged viral vector under conditions permitting the uptake of said packaged viral vector by said cells and expression of said polypeptide therein;

whereby increased permeability of said epithelial tissue facilitates improved viral transduction of said cells, which in turn facilitates expression of said polypeptide.

51. The method of claim 50, further comprising increasing the proliferation of cells of said epithelial tissue.
52. The method of claim 50, wherein said viral vector is a retroviral vector.
53. A method for treating an epithelial tissue disease comprising:
- (a) providing a packaged viral vector comprising a polynucleotide encoding said therapeutic polypeptide;

- (b) increasing the permeability of the diseased epithelial tissue; and
- (c) contacting cells of said epithelial tissue with said packaged viral vector under conditions permitting the uptake of said packaged viral vector by said cells and expression of said therapeutic polypeptide therein,

whereby expression of said therapeutic polypeptide treats said disease.

- 54. The method of claim 53, further comprising increasing the proliferation of cells of said diseased epithelial tissue.
- 55. The method of claim 53, wherein the diseased epithelial tissue is airway tissue.
- 56. The method of claim 55, wherein said diseased airway tissue is alveolar tissue, bronchial tissue or tracheal tissue.
- 57. (Withdrawn) The method of claim 53, wherein said disease is a cancer.
- 58. (Withdrawn) The method of claim 57, wherein said cancer is lung cancer.
- 59. (Withdrawn) The method of claim 57, wherein said cancer is tracheal cancer.
- 60. The method of claim 53, wherein said disease is an inherited genetic defect.
- 61. (Withdrawn) The method of claim 60, wherein said inherited genetic defect is surfactant protein B deficiency.
- 62. (Withdrawn) The method of claim 60, wherein said inherited genetic defect is alpha-1-antitrypsin deficiency.
- 63. The method of claim 60, wherein said inherited genetic defect is cystic fibrosis.

64. The method of claim 53, wherein said therapeutic polypeptide is selected from the group consisting of a tumor suppressor, a cytokine, an enzyme, a toxin, a growth factor, a membrane channel, an inducer of apoptosis, a transcription factor, a hormone and a single chain antibody.
65. The method of claim 53, wherein increasing the permeability of the diseased epithelial tissue comprises contacting cells of said diseased epithelial tissue with a tissue permeabilizing agent.
66. The method of claim 54, wherein increasing the proliferation of cells of said diseased epithelial tissue comprises contacting said cells with a proliferative agent.
67. The method of claim 53, wherein said viral vector is a retroviral vector.
68. A composition comprising EGTA and in a hypotonic solution.
69. The composition of claim 68, further comprising a packaged viral vector.
70. A method for transforming epithelial cells with a viral vector comprising delivering to said epithelial cells a packaged viral vector and EGTA in a hypotonic solution.

APPENDIX B: EXHIBITS

Retroviral Vectors Efficiently Transduce Basal and Secretory Airway Epithelial Cells *In Vitro* Resulting in Persistent Gene Expression in Organotypic Culture

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ABSTRACT

Gene therapy of the lung requires the introduction and expression of a therapeutic gene in airway cells. Although retroviral vectors may be useful in this context, the ability of retroviruses to infect specific cell types in the airway is not known. In this study, we examined the ability of amphotropic recombinant retroviral vectors to transduce primary cultures of rabbit airway epithelial cell populations purified for basal or secretory cells. Transduction efficiencies in basal and secretory cell populations were found to be similar; about 27% after a single exposure to vector, and up to 77% after multiple exposures. The fate of genetically modified cells from the different populations was followed through terminal differentiation using organotypic cultures. The epithelium of the organotypic cultures generated from each population exhibited both pseudostratified and stratified morphology, produced mucin, and stained positively with antibodies specific for basal and ciliated cells. The mucociliary epithelium also showed co-localization of these phenotypic markers with the expression of the vector-encoded β -galactosidase gene. We conclude that retroviruses can efficiently transduce primary cultures of basal and secretory cells, and that both of these cell types can be progenitor cells of the airway epithelium. *In vivo* delivery of a retroviral vector containing a human placental alkaline phosphatase gene resulted in expression of the heterologous gene in rabbit tracheal epithelial cells. However, transduction efficiency was low and occurred only in the wounded trachea.

OVERVIEW SUMMARY

We assessed the ability of retroviral vectors to transduce purified basal and secretory populations of airway epithelial cells and followed the fate of the retrovirally marked cells. We found that retroviral vectors efficiently delivered marker genes to both basal or secretory cell populations in monolayer cultures and that expression of the gene stably persisted in the epithelium generated in organotypic culture. Progeny basal, secretory, and ciliated cells in the epithelium generated from each population expressed the marker gene. Although retroviral transduction was efficient *in vitro*, the intact normal airway epithelium was resistant to retroviral transduction. However, a low transduction rate was observed in the wounded epithelium.

INTRODUCTION

GENETIC DISEASES THAT AFFECT THE LUNG may be cured by the use of gene therapy. Among these diseases, cystic fibrosis (CF) affects one in 2,500 newborns and leads eventually to respiratory failure and premature death. The gene responsible for CF is located on chromosome 7, spans 250 kb, and produces a 6.5-kb transcript that encodes the CF transmembrane conductance regulator (CFTR) (Riordan *et al.*, 1989). Transfer of the normal CFTR cDNA by retrovirus or vaccinia virus expression vectors into cultured CF epithelial cells can correct the cAMP-stimulated chloride ion channel defect (Drumm *et al.*, 1990; Rich *et al.*, 1990), indicating that gene therapy for CF may be possible. Little is known about the role or roles of CFTR within the various epithelial cell populations of the airway.

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Indeed, it is not known which cells are effectors of CFTR chloride channel function although CFTR expression is found in the serous cells (Engelhardt *et al.*, 1992) and the ciliated cells (Yankaskas *et al.*, 1993). Therefore, gene transfer to cells that give rise to all epithelial cell populations is one possible strategy for CF gene therapy.

Vehicles for gene transfer to the airway epithelial cells based on virus vectors have been explored aggressively (Rosenfeld *et al.*, 1992; Flotte *et al.*, 1993). In addition, liposome-mediated and receptor-mediated delivery have also been investigated (Alton *et al.*, 1993; Hyde *et al.*, 1993). Retroviral vectors, by virtue of their ability to integrate into the cell genome, could provide an effective long-term gene therapy for genetic diseases of the lung epithelium if appropriate self-renewing, multipotent epithelial cell populations could be targeted.

Historically, basal cells of the airway epithelium are thought to be the progenitor cells of the airway. *In vitro* studies using rat tracheal cells show that populations of cells positive for phenotypic markers for basal cells were multipotent epithelial progenitors (Liu *et al.*, 1994). However, studies in hamster trachea provide evidence for the important role of secretory cells in the regeneration of the epithelium following injury (Keenan *et al.*, 1983). The results of these studies suggest that both cell types can be airway epithelial progenitor cells. Delivery of therapeutic genes to these cell populations would result in gene transfer to all other cell types in the airway.

The purpose of this study was to assess the ability of retroviral vectors to transduce purified basal and secretory populations of airway epithelial cells and to follow the fate of the retrovirally marked cells. Growth of the transduced cells in organotypic cultures allowed analysis of the continued expression of transferred genes in differentiated cells and elucidated progenitor-progeny relationships in the airway epithelium. We found that retroviral vectors efficiently delivered marker genes into both basal or secretory cell populations *in vitro* and that expression of the genes was found in basal, secretory, and ciliated cells in the epithelium generated from each population. Having established that retroviral transduction was efficient *in vitro*, we next evaluated the performance of retrovirus vectors *in vivo*. We found that *in vivo* delivery into rabbit airways resulted in no detectable transduction in the intact epithelium and a low transduction rate in the wounded epithelium.

MATERIALS AND METHODS

Construction and generation of amphotropic retrovirus vectors

We constructed a retrovirus vector that encoded the nuclear localizing bacterial β -galactosidase (β -Gal) gene, LgZnSN, as follows. The β -Gal gene that contained a simian virus 40 (SV40) nuclear localization signal was isolated from the plasmid, pPD16.43 (Fire *et al.*, 1990), by digestion with *Bam* HI and *Stu* I restriction enzymes. The *Bam* HI site was made blunt using the Klenow fragment of DNA polymerase I, and the isolated fragment was inserted into the blunted *Xho* I site of the plasmid pLgXSN. LgXSN is a derivative of the Moloney murine leukemia virus (MoMLV) based retroviral vector LXS (Miller and Rosman, 1989) in which the retroviral proline tRNA primer binding site that is found adjacent to the long terminal

repeat (LTR) of the wild-type virus was changed to a glutamine tRNA primer binding site (C. Halbert, T. Palmer, and A.D. Miller, unpublished). In LgZnSN, the β -Gal gene is expressed from the MoMLV promoter/enhancer. This vector also contains a neomycin phosphotransferase gene expressed from the SV40 early promoter. The construction of the plasmids pLNPOZ and pLAPSN has been described previously (Adam *et al.*, 1991; Miller *et al.*, 1994). The retrovirus vector LNPOZ contains neomycin phosphotransferase and β -Gal genes that are transcribed from the MoMLV promoter. In LNPOZ, the galactosidase protein is cytoplasmic and is translated from a poliovirus internal ribosome entry site. The retrovirus vector LAPSN contains a neomycin phosphotransferase gene that is transcribed from a SV40 promoter. In LAPSN, the human placental alkaline phosphatase (AP) cDNA is expressed from the MoMLV promoter and enhancer. The retrovirus vectors are shown in Fig. 1.

Amphotropic retrovirus vectors were generated in the packaging cell line PA317 (Miller and Buttimore, 1986). In one *in vivo* experiment, the retrovirus vector LAPSN was packaged in a hybrid retrovirus packaging cell line (PG13) that expressed the gibbon ape leukemia virus envelope and MoMLV capsid protein (Miller *et al.*, 1991). Virus stocks were prepared as described (Miller and Rosman, 1989), except that virus collection was done in the keratinocyte serum-free growth medium (SFM; GIBCO BRL, Grand Island, NY) to produce virus stocks more suitable for use in epithelial cultures. Vector titers were determined by G418 selection in thymidine kinase deficient (TK⁻) NIH-3T3 cells (Miller and Rosman, 1989). The packaging cell lines PA317 and PG13 were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL) with high glucose (4.5 grams/liter) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), penicillin, streptomycin and amphotericin B.

Preparation of tracheal epithelial cell populations

Tracheal epithelial cells from adult New Zealand white rabbits were isolated as previously described (Aitken *et al.*, 1991). Briefly, tracheas were excised, rinsed in sterile Hanks' balanced salt solution (HBSS), and cut into pieces of approximately 10 mm². The tracheal mucosa was placed in 0.15% bovine testicular hyaluronidase (Sigma, St. Louis, MO) for 40 min at 22°C. The mucosa was then placed in 0.1% Pronase (Sigma, St. Louis, MO) at 37°C for 30 min. After exposure to Pronase, the epithelium was peeled from the mucosa. A single-cell suspension was obtained by placing the epithelial sheets in Tyrode's balanced salt solution (Sigma, St. Louis, MO) containing 0.1% trypsin (Difco Inc., Detroit, MI) for 5 min and then passed nine times through a 25-gauge needle. Approximately 10⁷ individual viable cells were obtained from each trachea. Epithelial cells were then placed in HBSS containing 1% bovine serum albumin (Sigma) and used for flow cytometry.

Flow cytometry

Cells were sorted as described previously (Aitken *et al.*, 1991). Briefly, the cells were suspended in HBSS and 1% bovine serum albumin and were sorted on an Orthocytofluorograph 50111 with a model 2150 computer (Ortho Diagnostic Systems, Westwood, MA). A model 164-01 krypton ion laser (Spectra Physics) was used for excitation of cells at 488 nm (180 mW). The morphologically different cells were sorted on the basis of variation of

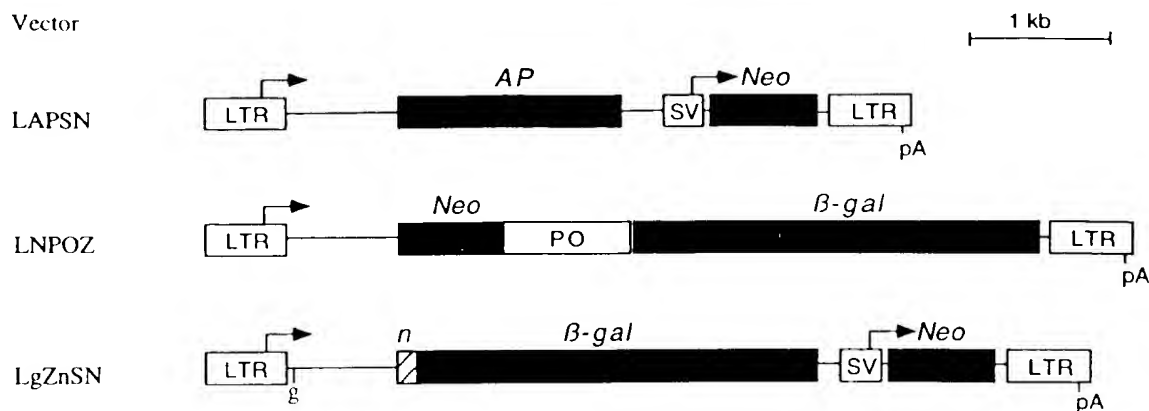


FIG. 1. Retroviral vectors. Abbreviations and symbols are as follows. LTR, Retroviral long terminal repeat; g, glutamine tRNA primer binding site; SV, SV40 early promoter and enhancers; PO, poliovirus internal ribosome entry site; AP, human placental alkaline phosphatase; Neo, neomycin phosphotransferase; β -gal, β -galactosidase; n, SV40 T antigen nuclear-targeting sequence; pA, polyadenylation signal. Arrows indicate promoters; filled-in boxes indicate protein coding regions, open boxes indicate the retroviral long terminal repeat, and lines indicate human and bacterial noncoding sequences and viral sequences other than the LTR, including the extended virus packaging signal that follows the retroviral LTR. Vector names are based on the order of genetic element within the vector.

the forward and right-angle scatter using a 100- μ m orifice and sorting rates of 1,500 cells/sec. Cells were recovered by centrifugation ($200 \times g$, 5 min) and resuspended in HBSS. Bivariate data were displayed using the program CONTOUR.

Flow cytometry yielded three different cell populations enriched for secretory, basal, and ciliated cells, respectively. The cellular composition of the different fractions obtained from flow cytometry was determined by light microscopy, and the typical appearance of the cells in the three different fractions has been examined by transmission electron microscopy (Aitken *et al.*, 1991). The purity of the fractions was confirmed for each experiment in this study by morphologic examination using light microscopy. A random sample representing 200–300 cells was visualized for size and presence of cilia for every cell sort. When populations were observed to be <97% pure, the flow cytometer gates were readjusted to achieve the required purity of >97% basal or >97% secretory cells by light microscopic examination. We do not know the identity of the contaminating cells in the basal population. The contaminating cells in the secretory population tend to be ciliated cells that do not attach in single-cell suspensions, and therefore do not contribute to the progeny population.

The viability of the cells was examined using 4'-6-diamidino-2-phenylindole stain and Hoechst 33258 stain. Using flow cytometry to assess fluorescence, the viability of the epithelial cells was >98% before sorting and 90% after sorting. The attachment rate was $19.3 \pm 6\%$ (SD) for the basal cell fraction ($n = 3$) and $29.0 \pm 1.4\%$ (SD) for the secretory cell fraction ($n = 3$), while the ciliated cells did not attach. Approximately 2.5×10^5 basal and 3.0×10^5 secretory cells were obtained from each trachea after 60 min of flow sorting.

Retrovirus infection

Purified basal and secretory cells were seeded into six-well culture dishes at $1-2 \times 10^5$ cells/well and allowed to attach

overnight. The next day, cells were infected by incubation with 1 ml of virus mixed with 1 ml of SFM and Polybrene (4 μ g/ml; Sigma). The virus inoculum was removed after 6 hr, and the cells were rinsed in phosphate-buffered saline (PBS) and refed with SFM. Cells were stained for β -Gal or AP expression 2 or 5 days post infection, or were used to generate organotypic cultures at day 7. Cells that were infected for 3 consecutive days were treated with recombinant virus for 6–8 hr each day. Determination of the percentage of transduced cells in monolayer cultures was done by counting at least 10 random fields representing 400–1,300 cells.

Organotypic cultures

Primary rabbit tracheal epithelial populations obtained by flow cytometry were plated in six-well dishes coated with vitrogen (Celtrix Pharmaceuticals, Santa Clara, CA), and grown in SFM. Cultures were transduced by retroviral vectors, LNPOZ(PA317) and LgZnSN(PA317), within 24 hr after cell isolation. Cells were used to generate organotypic cultures after growth in monolayers for 7 days. Primary rabbit tracheal fibroblasts used in organotypic cultures were isolated from the rabbit trachea after removal of the epithelial layer. The submucosa was digested by an overnight incubation in DMEM containing 10% FBS, antibiotics, and collagenase (200 U/ml; Worthington Biochemical). Clumps of cells were washed in PBS and pelleted by centrifugation, treated with 1% trypsin and ethylenediaminetetraacetic acid for 10 min at 37°C, passed 20 times through a 2-ml pipet, and pelleted again by centrifugation. Cells were resuspended in DMEM supplemented with 10% FBS, penicillin, and streptomycin, and grown in tissue culture monolayers. Although epithelial cells from the submucosa also attached, these represented approximately 1% of the initial cell population and were lost with subsequent passaging. Fibroblasts at passages 2–5 with a split ratio of 1:3 were used in the organotypic cultures.

Organotypic cultures were made using a modification of previously published procedures (McCance *et al.*, 1988; Halbert *et al.*, 1992). A dermal-equivalent collagen layer was prepared as described by the manufacturer (Collaborative Research Inc., Bedford, MA); 5×10^5 primary rabbit tracheal fibroblasts obtained by trypsinization of monolayer cultures of fibroblasts were mixed with collagen, polymerization buffer, and nutrient medium to form a thick collagen matrix (one part DMEM to three parts F12 nutrient medium (GIBCO BRL), 4.5 mg of collagen, neutralization buffer, 5% FBS, and antibiotics) in a 35-mm dish. Then, 7-day monolayer cultures of epithelial cells (transduced by retroviral vectors on day 1) were trypsinized and resuspended in DMEM containing 5% FBS. Approximately 2.5×10^5 epithelial cells were seeded on top of the collagen gel. The next day, the collagen gel was dislodged from the edge and bottom of the plate to allow the collagen to contract. The cultures were grown submerged for 5 days and then raised to an air-liquid interphase to continue their growth for an additional 16 days. Cultures were fed daily with DMEM supplemented with 5% FBS and growth factors (Halbert *et al.*, 1992). The lifted cultures were fed the same medium plus 5×10^{-8} M retinoic acid.

Staining for β -Gal and AP expression

Monolayer cultures were rinsed in PBS, fixed in 3.7% formaldehyde in PBS for 15 min, and rinsed three times in PBS. Staining for β -Gal expression was done by immersing fixed cultures in reaction buffer (25 mM $K_3Fe(CN)_6$, 25 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, and 1 mg/ml of 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside in PBS) for several hours at 37°C or overnight at room temperature. Organotypic cultures were rinsed in PBS, fixed in PBS containing 4% paraformaldehyde for 30 min, and then rinsed three times in PBS. After overnight staining in β -Gal reaction buffer at room temperature, the organotypic cultures were fixed in PBS containing 4% paraformaldehyde for an additional 90 min. Sections were cut from paraffin-embedded samples.

The procedure for AP staining of cells in monolayers has been previously described (Halbert *et al.*, 1994). AP staining of rabbit trachea was as follows. The excised tracheas were fixed overnight in 3.7% formaldehyde in PBS at room temperature, then washed three times (90 min each) in PBS. Afterwards, the tracheas were incubated in PBS at 65°C for 90 min (to eliminate endogenous AP activity) and were then incubated overnight at room temperature in staining buffer (100 mM Tris pH 8.5, 100 mM NaCl, 50 mM $MgCl_2$, 1 mg/ml of nitro blue tetrazolium, 0.1 mg/ml of 5-bromo-4-chloro-3-indolylphosphate).

Immunohistochemistry

The 5B4/H3 monoclonal antibody that recognized ciliated cells was obtained by immunizing mice with rabbit tracheal epithelium (Aitken *et al.*, 1993). The 1D9/B3 monoclonal antibody that stained basal cells was obtained by immunizing mice with purified tracheal epithelial basal cells (Aitken *et al.*, 1995).

Sections of organotypic culture were preincubated with 1% normal goat serum in Tris-buffered saline (TBS) (0.15 M NaCl

in 0.05 M Tris pH 7.5) for 60 min at room temperature. The basal cell or ciliated cell antibodies were then applied to the sections overnight at 4°C. Sections were rinsed with TBS containing 0.05% Tween20. Sections were then incubated with peroxidase-conjugated goat anti-mouse IgA/IgG/IgM (1:200) for 1 hr, rinsed three times in TBS, then developed with 30 ml of TBS containing 0.3% diaminobenzidine and 50 μ l of 30% H_2O_2 for 2 min at room temperature, followed by a rinse with TBS containing 0.05% Tween20 and mounted. Positive controls used in the monoclonal antibody staining included tracheal sections that stained positive for both the basal cell and ciliated cell antibodies. Negative controls included organotypic culture sections exposed to preimmune serum only, secondary antibody only, or type-matched primary antibodies.

In vivo delivery of retroviral vectors

Animal procedures were approved by the Animal Care Committee of the University of Washington. New Zealand white rabbits were anesthetized by an intramuscular injection of ketamine (Aveco Co Inc, Fort Dodge Labs, IA) and xylazine (Lloyd Labs, Shenandoah, IA).

In one experiment, the rabbits were intubated endotracheally after anesthesia. Two animals received treatment with a bronchial cytology brush (diameter, 2 mm; Mill-Rose Laboratories, Mentor, OH). The trachea was brushed distal to the end of the endotracheal tube. On the left side, the left main stem bronchus and the segmental bronchus of the left lower lobe were brushed. On the right side, the right main stem bronchus, the bronchus intermedius, and the segmental bronchus of the right lower lobe were brushed. Because brushing was not performed under direct fiberoptic visualization, the damage to the epithelium was not expected to be uniform. The brush was passed 10 times in all, brushing both on entry to and exit from the airways. Immediately after brushing, approximately 2×10^8 cfu of LAPS(NPA317), in a 3-ml volume, was instilled into the trachea of each of 2 rabbits (one having a wounded trachea due to abrasion with a bronchial brush). Another brushed animal was given culture medium (DMEM supplemented with 10% FBS and antibiotics). The virus stock was concentrated as previously described (Paul *et al.*, 1993).

In another experiment, 3 animals were anesthetized, and retroviral vectors were aerosolized into the trachea and lung using an Acorn 2 jet nebulizer (Marquest, Englewood, CO) powered by a Devilbiss power source. Environmental contamination was prevented by placing double filters (Pall Filters, Model BB50T, Hank Medical, Seattle, WA) in the exhalation port of the ventilator circuit. Six milliliters of a retroviral vector, LAPS(NPA317) (3×10^8 cfu as determined by G418 selection in TK⁻/NIH-3T3 cells) or LAPS(NPG13) (4.2×10^6 cfu as determined by G418 selection in HeLa cells) were aerosolized into each of 2 animals. The vector produced from the PG13 packaging cell line was included because it transduced monolayer cultures of rabbit primary airway epithelial cells two-fold more efficiently than the vector produced from the PA317 packaging cell line (data not shown). The third animal received aerosolized tissue culture medium. Total aerosolization time was approximately 15 min. Because retroviruses do not withstand prolonged desiccation as may happen during aerosoliza-

tion, the infectivity rate by nebulization may be less than anticipated from the initial viral titer. To determine the loss in virus titer after nebulization, aerosolized virus was collected by condensation from the end of the tubing attached to the nebulizer. The titer after nebulization was 50% of the starting titer.

Infected and control animals were sacrificed at day 7 post infection using intravenous pentobarbitone, and the excised tracheas were processed for AP staining. In the first experiment (brushed versus nonbrushed trachea), comparison was made between vector-treated animals and medium-treated control animal. Portions of the trachea where AP stain could be seen in a dissecting microscope (in the vector-treated and bronchial-brushed animal) and portions of the trachea corresponding to the same anatomical site in the other animals were taken for histologic examination. In the second experiment (aerosolized vector), tissue samples representing portions of the trachea from proximal to distal were taken for histologic examination. The tissue samples were paraffin-embedded and the tissue sections were stained with Nuclear Fast Red. Quantitation of transduction efficiency was done by counting the number of AP-positive epithelial cells, dividing by the total number of epithelial cells, and expressing the result as a percentage.

RESULTS

Efficient transduction of basal and secretory cell populations by retroviral vectors

Purified epithelial cell populations were transduced within 24 hr after isolation with amphotropic retroviral vectors carrying the β -Gal gene, LNPOZ(PA317) and LgZnSN(PA317) (2×10^5 cfu/ml as determined by using NIH-3T3 cells), or the human alkaline phosphatase gene, LAPS(PA317) (5×10^6 cfu/ml as assayed in NIH-3T3 cells). Table 1 shows the percentage of cells that exhibited staining for β -Gal or AP expression. Ciliated cells were not analyzed because single-cell suspensions of this population did not attach to tissue culture plates. Transduction of primary cultures of basal and secretory cell populations with retroviruses yielded similar percentages of positive cells, indicating that both cell types were equally transduced by the amphotropic retroviral vectors. Transduction efficiency ranging from 4.5% to 6.3% was obtained using LNPOZ(PA317) and LgZnSN(PA317). Higher transduction efficiency (26–28%) was obtained by using LAPS(PA317), a

result that was probably due to the higher titer of this vector compared to those of the β -Gal vectors. Transduction efficiency of 66% and 77% was achieved in secretory and basal cells, respectively, upon daily administration of 1 ml of PA317/LAPS virus for 3 days after isolation ($n = 1$).

Transduced cells continue to express marker gene during terminal differentiation

The epithelium generated in the organotypic cultures derived from basal and secretory populations transduced by the retrovirus vector LNPOZ(PA317) contained cells that continued to express β -Gal activity during terminal differentiation (Fig. 2). β -Gal positivity in the epithelium derived from the secretory population was between 8% and 9% after 12 or 21 days in organotypic cultures, (Table 2). Similarly, β -Gal positivity in the epithelium derived from the basal population was between 9% and 10% after 12 or 21 days, (Table 2). These results show that there was no reduction in the percentage of β -Gal-positive cells during maturation of the epithelium (between 12 and 21 days).

Positive cells were often found in clusters, indicating clonal growth during maturation of the epithelium. Large clusters of β -Gal-positive cells as well as single β -Gal-positive cells occurred in both populations (Fig. 2, compare upper and lower right panels), demonstrating that a subset of the population transduced by retrovirus vectors are progenitor cells having a large expansion capacity. The morphology of the epithelium spanned a spectrum from stratified to pseudostratified at earlier time points (Fig. 2, day-12 panels), and progressed to one that was stratified, pseudostratified and columnar at maturation (Fig. 2, day 21 panels). The appearance of ciliated cells was more pronounced in the later cultures, indicating that progenitors of these cells within each population have achieved terminal differentiation by day 21. The time course of maturation of the epithelium varied slightly dependent on the density of the epithelial cells seeded onto the collagen matrix. The higher density seeding resulted in a shorter time required for maturation and ciliation of the epithelium.

Genetically modified cells express phenotypic markers of basal, secretory, and ciliated cells

Binding of antibodies directed against basal and ciliated cells was examined in the epithelium to verify that genetically modified cells expressed antigens specific for these cell types in the

TABLE 1. INFECTION EFFICIENCY OF RETROVIRAL VECTORS IN POPULATIONS OF AIRWAY EPITHELIAL CELLS^a

Vector	Basal	Secretory
LNPOZ(PA317)	4.5 \pm 0.8% ($n = 2$)	5.8 \pm 3.1% ($n = 2$)
LgZnSN(PA317)	6.3 \pm 3.2% ($n = 4$)	5.9 \pm 2.5% ($n = 4$)
LAPS(PA317)	28.0 \pm 4.0% ($n = 2$)	26.0 \pm 1.4% ($n = 2$)

^aPrimary cultures of airway epithelial cell types were infected with recombinant retroviruses within 24 hr after isolation. Cells were stained for β -Gal or AP at 48 hr (LgZnSN and LAPS) or 5 days (LNPOZ) after transduction. Values in the table represent the percent of the cell population staining for positive β -Gal or AP expression, \pm SD.

TABLE 2. β -GAL POSITIVITY IN THE EPITHELIUM OF THE ORGANOTYPIC CULTURES GENERATED FROM BASAL AND SECRETORY CELL POPULATIONS^a

Cell	Day 12	Day 21
Basal	9.9 \pm 3.5% (n = 2) ^b	9.0 \pm 5.0% (n = 2) ^c
Secretory	8.9 \pm 2.4% (n = 3) ^d	7.7 \pm 1.5% (n = 3) ^e

^aMonolayer cultures of cells were transduced with the retroviral vector PA317/LgZnSN within 24 hr of isolation of basal and secretory cells from rabbit trachea. After 7 days in monolayer cultures, cells were placed in organotypic cultures. β -Gal positivity was determined after 12 and 21 days in organotypic culture. Values in the table represent the percent of the cell population staining positive for β -Gal expression, \pm SD.

^bSix sections were examined and 6.3×10^3 cells counted to determine percent positivity.

^cEight sections were examined and 7.6×10^3 cells counted.

^dSix sections were examined and 8.0×10^3 cells counted.

^eEight sections were examined and 1.3×10^4 cells counted.

airway. For this purpose, the basal and secretory cell populations were transduced with the vector that encoded the nuclear localizing β -Gal (LgZnSN) rather than the vector that encoded the cytoplasmic β -Gal (LNPOZ) because the cytoplasmic β -Gal stain interfered with the ability to detect antibody staining.

The 1D9/B3 basal cell antibody stained basal cells in organotypic cultures initiated from either basal or secretory cell populations (Fig. 3, top two panels). The staining was found at the base of the epithelium where the cells were adjacent to the fibroblast-collagen matrix. This region is where basal cells are found *in vivo* and it is also the region where the 1D9/B3 basal cell antibody staining is localized *in vivo* (Aitken *et al.*, 1995). We also observed some staining of the fibroblasts in the collagen matrix with this antibody, although staining of fibroblasts in rabbit trachea sections has not been observed (Aitken *et al.*, 1995). This may be due to a cross-reacting antigen found in the cultured fibroblasts. The β -Gal staining showed that basal and secretory cells transduced by retrovirus vectors expressed the heterologous gene product in progeny basal cells derived from each population.

The 5B4/H3 ciliated cell antibody stained cells in the organ-

otypic cultures derived from the secretory as well as the basal cell populations (Fig. 3, middle two panels). Staining was confined to the apical surface of the epithelium and within the lumen of intercellular vacuoles found within the epithelium generated from either populations. Only cells which exhibited cilia were stained with 5B4/H3. We have observed more staining by the ciliated cell-specific antibody in the day 21 cultures (Fig. 3 middle two panels) than in the day 12 cultures (data not shown). The arrows show co-localization of β -Gal and ciliated cell antibody staining.

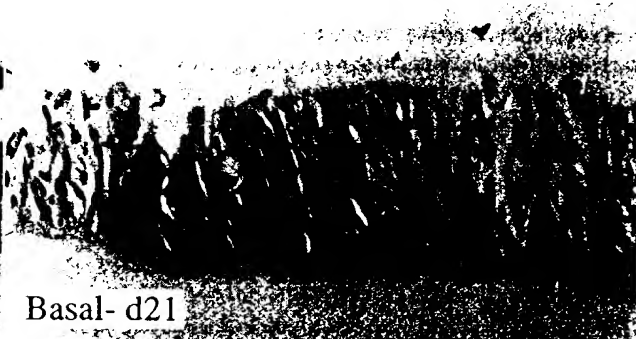
A histochemical stain, mucicarmine, was used to identify secretory cells because incubation of the *in vitro* epithelium (as well as rabbit trachea) with a secretory cell antibody gave a high background staining. Intracellular and intercellular vacuoles within the epithelium of the organotypic culture stained positively for mucin as well as β -Gal staining nuclei (Fig. 3, lower panels). The results show that basal and secretory cell populations yielded mucin-producing cells, demonstrating that both cell populations contained progenitors of secretory cells. Together, Figs. 2 and 3 show that the growth of cells from both basal and secretory populations generated an epithelium in

FIG. 2. Expression of the β -Gal protein in differentiated epithelial cells from basal and secretory cell populations. Airway epithelial cells transduced by LNPOZ(PA317) were grown in organotypic cultures for 12 or 21 days, fixed in paraformaldehyde and stained for β -Gal expression. Sections of the epithelium were counterstained with Nuclear Fast Red. The morphology of the epithelium generated from basal and secretory cell populations was stratified, pseudostratified at day 12 (Fig. 2, left panels) and progressed to one that was stratified, pseudostratified, and columnar at day 21 (Fig. 2, right panels). The photomicrographs are examples of the spectrum of morphologies found in the *in vitro* epithelium and the pattern of β -Gal staining observed. Although the upper panels were derived from a basal cell population and the lower panels were derived from a secretory cell population, β -Gal-stained cells ranging from a single cell to a large cluster of cells could be found in the epithelium generated from both cell populations. Original magnification, 400 \times .

FIG. 3. Basal and secretory cell populations are progenitors of basal, secretory, and ciliated cells. Cell populations were transduced by the retroviral vector, LgZnSN(PA317). Sections of paraformaldehyde-fixed and β -Gal-stained organotypic cultures were reacted with antibodies specific for basal cells (1D9/B3, top panels) and ciliated cells (5B4/H3, middle panels). The epithelium was also stained for mucin expression (mucicarmine, bottom panels). The arrows mark cells where positive staining with antibodies or staining for mucin co-localized with β -Gal expression. Panels showing staining for basal cells were done on day-12 cultures whereas panels showing staining for ciliated cells and secretory cells were done on day 21. The epithelium generated from both progenitor populations yielded basal, ciliated and secretory cells. Original magnification, 400 \times .



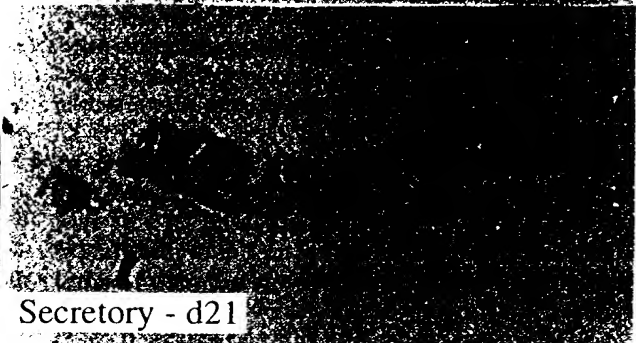
Basal - d12



Basal- d21



Secretory - d12



Secretory - d21

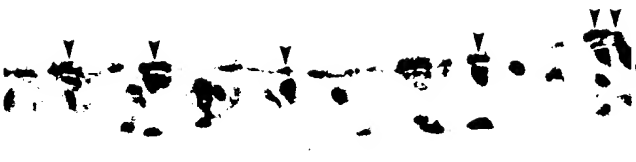
FIG. 2



Basal - 1D9/B3



Secretory - 1D9/B3



Basal - 5B4/H3



Secretory - 5B4/H3



Basal - mucicarmine



Secretory - mucicarmine

FIG. 3

organotypic culture that was reminiscent of the airway, and contained basal, ciliated and mucin-producing cells. Progenitor cells marked by a retrovirus vector continued to express the heterologous gene product in their progeny through terminal differentiation.

A retroviral vector can transduce epithelial cells in wounded rabbit trachea in vivo but with poor efficiency

The transduction rate observed for cultured airway epithelial cells showed that under proliferative conditions retroviral transduction can be efficient. Therefore, retroviral transduction in normal and wounded rabbit trachea *in vivo* was examined. We reasoned that stimulating a wound healing process would result in an increase in epithelial proliferation, and concomitantly facilitate retroviral transduction because these retroviral vectors appear to primarily transduce dividing cells (Miller *et al.*, 1990). LAPSN(PA317) (2×10^8 cfu as assayed by using NIH-3T3 cells) was delivered to the upper airways of each of two rabbits. The airway of one of the two rabbits was abraded with a bronchial brush prior to vector delivery. A control rabbit was similarly abraded but received culture medium without vector (DMEM supplemented with 10% FBS and antibiotics). Seven days after

virus instillation, the animals were euthanized and the tracheas were processed for AP staining. Only the animal treated with the virus vector in conjunction with tracheal brushing showed AP staining of epithelial cells (Fig. 4b-d). Morphologically, the transduced cells appear to be basal (Fig. 4c), ciliated (Fig. 4b) and secretory cells (Fig. 4d). The epithelium from the control rabbit that received culture medium (Fig. 4a) and from the rabbit that received vector without tracheal brushing (data not shown) did not exhibit AP staining of epithelial cells. In a later experiment, tracheas of rabbits that received a β -Gal retrovirus vector LgZnSN (PA317) were stained for AP. No AP staining of epithelial cells in the trachea was observed in these vector control animals (data not shown). In 10 slides containing 90 sections of tracheal epithelium and representing 2×10^5 epithelial cells, 20 AP-positive epithelial cells were found. This represented a low transduction efficiency of 0.01%. In the nonbrushed, vector-treated animal, no AP-positive cells were detected in 45 sections representing 10^5 cells (transduction efficiency <0.001%).

The anatomical site of the trachea would not facilitate prolonged exposure to vector because most of the inoculum would flow quickly downward into the lung. We explored the possibility that aerosolization of the vector may allow a more even distribution of the virus particles in the proximal airways and result in an increase in transduction of the epithelium. The vec-

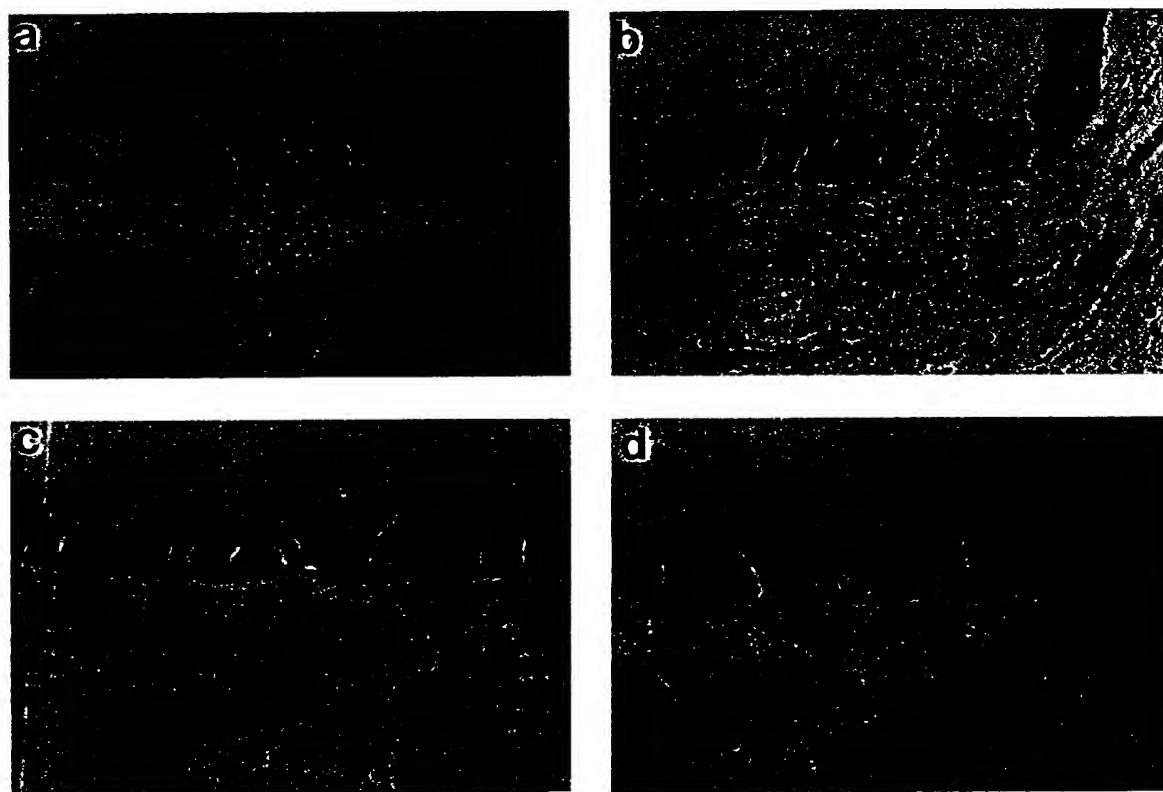


FIG. 4. Transduction of airway epithelial cells *in vivo* by retroviral vector. Rabbit tracheas were inoculated with culture medium or amphotropic retroviral vector LAPSN(PA317) after treatment with a bronchial brush. The tracheas were stained for AP and sections of the tissues were counterstained with Nuclear Fast Red. The trachea from the control rabbit (Fig. 4a) did not show AP staining of epithelial cells, whereas the trachea of the rabbit inoculated with PA317/LAPSN had AP-positive epithelial cells (Fig. 4b-d). These cells appear to be basal (c), ciliated (b), and secretory (d) in morphology. Original magnification, 400 \times .

tors LAPSN(PA317) (3.8×10^8 cfu as assayed in NIH-3T3 cells) or LAPSN(PG13) (4.2×10^6 cfu as assayed in HeLa cells), or tissue culture medium, were aerosolized into each of 3 animals, respectively. The tracheas of these animals were not abraded prior to vector delivery. The animals were euthanized 7 days post infection and their tracheas were processed for AP staining. We did not detect any AP positivity in the tracheal epithelium by gross examination using a dissection microscope. Histologic sections taken of representative tissues from each trachea (sections from six to nine tissue samples totaling from 1.6 to 2.4×10^4 tracheal epithelial cells/animal) also did not show any transduced epithelial cells by AP staining (transduction efficiency $<0.005\%$).

DISCUSSION

We have shown that amphotropic retroviral vectors can efficiently transduce basal and secretory airway epithelial cell populations *in vitro* and that expression of the retrovirally transferred genes persisted through terminal differentiation in both populations. High transduction efficiencies were possible when the high-titer virus LAPSN(PA317) was used.

We have developed an organotypic culture procedure for growing airway epithelial cells. Our method is a modification of one used previously for the culture and differentiation of keratinizing squamous epithelial cells (McCance *et al.*, 1988; Halbert *et al.*, 1992). Other studies have used collagen substrates and growth at the air-liquid interface for culturing airway epithelial cells (Yankaskas *et al.*, 1985; Adler *et al.*, 1987; Chevillard *et al.*, 1993). We have found that incorporation of tracheal fibroblasts within the collagen matrix, in combination with medium enriched in serum and growth factors, more consistently generated an epithelium reminiscent of the *in vivo* airway. In comparison to the rat tracheal graft model (Engelhardt *et al.*, 1991), the organotypic culture system offers greater accessibility for manipulation of environment conditions. This culture system may provide a useful model for the study of the differentiation of airway epithelial cells and for the study of the ability of gene transfer vectors to transduce a differentiated epithelium.

The organotypic culture of airway epithelial cell populations demonstrated that the progeny of either basal or secretory cells could generate a differentiated epithelium that contained retrovirally marked basal, secretory, and ciliated cells, indicating that both basal and secretory cells can be progenitors of the three major cell types in the airway. A study using bulk populations of primary airway epithelial cells showed that a retroviral vector had transduced progenitors of ciliated and secretory cells (Engelhardt *et al.*, 1991). Our study demonstrated that the progenitor cells can be either of basal or secretory origin. Our results showing that rabbit basal and secretory epithelial cell populations have pluripotent capability are consistent with published studies done in hamsters and rats (Donnelly *et al.*, 1982; Breuer *et al.*, 1990; Johnson *et al.*, 1990; Randell *et al.*, 1991). Cellular targets for gene therapy using recombinant retrovirus can then include secretory as well as basal cells because each population can regenerate a mucociliary epithelium. However, it is still possible that one or the other may prove to be a better target for long-term gene therapy because of its greater proliferation capacity.

The behavior of basal and secretory cell populations suggests that the manipulation and culturing of cells may allow the cells to reprogram their differentiation potential. We have noted that a considerable fraction of the secretory cells degranulate during flow cytometry (Aitken *et al.*, 1995), and that, although the basal and secretory cell populations were sorted by size and retained their size differences at 24 hr after isolation, the basal cells enlarged slightly after attachment so that the basal and secretory cell subpopulation appeared similar in morphology and size after 2 days in culture. Indeed, the organotypic cultures generated from each population were indistinguishable from each other. The basal cells and the secretory cells proliferate *in vitro* to give rise to progeny that can become any of the three major cell types of the airway when placed into a differentiation environment. Other researchers (Terzaghi *et al.*, 1978; Chang *et al.*, 1985; Wu *et al.*, 1990) have also noted phenotypic changes of cultured tracheal cells. The observations obtained from our study and these previous ones are consistent with the idea of the plasticity of airway epithelial cells.

In vivo infection of airway epithelial cells by retroviral vectors is predicted to be less efficient presumably due to the much lower proliferation rate *in vivo* and the inaccessibility of the proliferating basal and secretory cells. Thus, it was not surprising that we did not detect any transduction *in vivo* after delivery of LAPSN(PA317) into normal rabbit airway either by direct instillation or aerosolization. Our *in vitro* study showed that in the proper environment basal and secretory cells can be efficiently transduced by retrovirus vectors. Indeed, *in vivo* transduction of epithelial cells was possible after wounding the trachea. However, the transduction rate was still low. There may be other factors in addition to proliferation that affect retroviral vector transduction in the airway epithelium. The number of receptors may be low *in vivo* and their localization inaccessible to the vectors. Unfortunately, antibodies to the receptors that can be utilized *in situ* were not available.

Present efforts in gene therapy directed to the airway epithelium have emphasized the use of adenovirus vectors. However, major problems with immune response and transient expression have demonstrated the need for further improvement before this vector can be used effectively and safely. Additionally, although the adenoviral vectors efficiently transduced airway cells *in vitro*, the efficiency differed dramatically *in vivo* because the columnar cells that comprise the major surface area of the airway epithelium were poorly transduced by adenovirus vectors (Grubb *et al.*, 1994). Adeno-associated viral vectors have been shown to give a high gene transfer rate (Flotte *et al.*, 1993). However, the transduction efficiency of adeno-associated virus as measured by gene expression appears to be low in primary cells (Halbert *et al.*, 1995) and in stationary cells (Russell *et al.*, 1994) and has yet to be fully characterized in the normal airway epithelium. We have shown that retroviral vectors can efficiently transduce basal and secretory airway epithelial cells in culture, where the cells are actively proliferating and accessible to vector. The *in vivo* results, although preliminary, suggest that a wound-healing environment enhances transduction by the retroviral vector. This may be due to a proliferation stimulus as well as a loss in integrity of the protective mucous layer due to abrasion of the epithelium. Together, the *in vitro* and *in vivo* data suggest that development of methods to stimulate division of the airway epithelium and to

deliver vectors to the more proliferative progenitor cells are important avenues to pursue.

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Bad for cats, good for humans? Modified feline immunodeficiency virus for gene therapy

Commentary

See related article,
pages R55-R62.

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When the concepts of gene therapy were evolving in the late 1980s, the focus was primarily on the hereditary disorders. This was a logical choice for the budding community of gene therapists, in that most hereditary disorders are autosomal recessive deficiency diseases that can theoretically be corrected by transfer of sufficient amounts of the normal gene to the cells manifesting the disease. This concept may be logical, but putting it into practice has been a real challenge. In this issue of the *JCI*, Wang et al. (1) describe the adaptation of feline immunodeficiency virus (FIV) to transfer the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) cDNA to the respiratory epithelium. This strategy represents a new approach to overcoming some of the challenges in using gene therapy to correct the respiratory manifestations of CF.

Unlike treatment for hematologic disorders, for which target cells for gene therapy can be removed from an individual and subjected to gene transfer manipulations in vitro, gene transfer to internal organs such as the lung requires the gene to be transferred in vivo. In treating the respiratory manifestations of CF, the targets for gene therapy are the 10^{10} epithelial cells lining the airways (2). Because of mutations in the 2 parental *CFTR* genes, the airway epithelial cells of individuals with CF have a deficiency of CFTR function, manifested by decreased Cl-transport in response to elevations of intracellular cAMP. It is estimated that correction of 5–10% of airway epithelial cells would be sufficient to prevent the clinical manifestations of CF. The level of normal gene expression required for each cell is not the problem, because the normal airway epithelium has an average of only 1–2 normal *CFTR* mRNA copies. However, because CF is a hereditary disorder, persistent expression of the newly transferred gene is essential for successful treatment.

To date, most investigators have focused their attention on 3 basic strategies for the transfer of the CFTR cDNA to the airway epithelium: use of adenovirus vectors, adeno-associated virus vectors, or plasmids combined with liposomes. All 3 strategies have been evaluated in humans with CF (3, 4–10). The adenovirus vectors are by far the most effective, providing the highest levels of gene expression in the respiratory epithelium of any gene vector system — levels that are sufficient to treat the disease (3). The challenge in the adenovirus strategy is that host defenses limit the persistence of expression (3, 11). Adeno-associated virus vectors have a theoretical advantage in that, if they can reach the nucleus of the target cell and be converted from their normal single-stranded DNA, they may be able to integrate into the host chromosome and thus provide long-term expression (10, 12, 13). Unfortunately, there seems to be a block in the lung epithelium at some step in this process, and these vectors, in their current form, yield clinically inadequate levels of expression. Likewise, the plasmid/liposome combinations are very inefficient, and expression in the lung is very low and only transient (6, 8).

From these observations, some rules have evolved that define the modifications to the current vector systems required to successfully treat respiratory manifestations of CF. If adenovirus vectors are to be used, they will have to be more efficient in entering the epithelium (so less vector can be used, thus limiting the extent of the host response to the vector), and/or the vectors will have to be designed to avoid triggering the adaptive and innate host defenses arrayed against the vector, a feature described as "stealth." If adeno-associated virus vectors are to be successful, strategies will have to be developed to bypass biological impediments to integration within the host genome.

Finally, if plasmids combined with liposomes are to be effective, signals will have to be built into the vector system to guide the plasmid into the cytoplasm, and then find, enter, and persist in the nucleus (14).

The study by Wang et al. (1) uses an alternative vector system that seems well-suited to the challenge of transferring and expressing the normal human CFTR cDNA in the differentiated airway epithelium. The strategy is based on FIV, a T-lymphotropic retrovirus first identified in 1987 in pet cats in Davis, California (see ref. 15 for a review of FIV). Based on its morphologic and biochemical characteristics, cell tropism, Mg^{2+} dependent reverse transcriptase, genetic organization, and antigenic properties, FIV is classified (along with HIV) as a lentivirus. Among the lentiviruses, FIV is most similar to the *Visna maedi* virus. FIV is 105–125 nm in diameter, spherical to ellipsoid in shape, with short, poorly defined projections on its envelope. The 9.4-kb FIV genome is organized like that of other retroviruses, with *env*, *gag*, and *pol* genes, with an additional 7 open reading frames that encode proteins. Wild-type FIV is tropic for T cells, B cells, macrophages, astrocytes, and microglia. Once it enters the cell, FIV has a life cycle similar to that of other retroviruses. Cats infected with FIV develop acute lymphadenopathy, neutropenia, and fever. As with HIV infection in humans, this is followed by an asymptomatic phase lasting years, after which generalized lymphadenopathy, recurrent fevers, apathy, leukopenia, anemia, anorexia, weight loss, stomatitis, and behavioral problems ensue. Finally, there is a terminal AIDS-like phase, marked by opportunistic infections, neoplasia, and neurologic abnormalities.

By having genes deleted that wild-type FIV requires to direct its own replication, can be converted into a replication-incompetent virus, capable of transferring recombinant cDNAs such

as CFTR, but incapable of reproducing except in a producer cell line that expresses the deleted genes (1, 16, 17). The concept of using a retrovirus for gene therapy is not new — the gene therapy field started with the idea of using modified forms of the Moloney murine leukemia virus (MMLV) to create a replication-deficient vector to transfer genes to target cells (14). The problem with using MMLV-based gene transfer vectors for CFTR gene transfer is that lung epithelial cells are slow to replicate, and the MMLV vectors require the target cell to proliferate for the viral genome with its recombinant gene to reach the target cell nucleus. Not so for FIV. For reasons that are not clearly understood, FIV is capable of delivering its genetic cargo to the nucleus of quiescent cells, where it randomly integrates into the host genome (15). For gene therapy for a hereditary disease, this is a clear advantage, because the newly delivered, integrated gene is permanently transferred from parent cells to daughter cells. In contrast, adenovirus vectors deliver their recombinant genomes to an extrachromosomal location, and thus when the target cell does divide, only 1 daughter cell is genetically modified (14).

To take advantage of the ability of FIV to infect slowly replicating human airway cells, another hurdle must be overcome — wild-type FIV does not infect humans. FIV primarily infects the modern domestic cat (*Felis catus*), and to a lesser extent large wild cats, but not other species (15). There is no evidence to link FIV to any human disease, and humans bitten by infected cats do not develop anti-FIV antibodies. Thus, an FIV gene transfer vector is useless for potential human gene transfer studies unless its coat is modified to interact with receptors on human cells. To circumvent this limitation, Wang et al. (1) modified the FIV vector to be pseudotyped with a vesicular stomatitis virus G (VSV-G) protein coat, a strategy that has been used to alter the host range of other retrovirus gene transfer vectors (16–18).

Does a replication-incompetent, VSV-G pseudotyped FIV vector solve the challenges of effectively transfecting human airway epithelial cells? The answer is . . . partially. The human airway epithelium is well designed to avoid viral infection, in that it hides most of the receptors that viruses use for entry —

including the receptor used by VSV-G — on its basolateral epithelial surface (19, 20). Thus, the FIV vector may be capable of inserting its gene into the host genome, but it cannot do this unless it can enter the cell. To overcome this obstacle, Wang et al. (1) transiently opened the tight junctions of the epithelium, using a formulation that was hypotonic and contained the calcium chelator EGTA (21–23). Together, the combination seems effective, with the VSV-G pseudotyped FIV vector capable of delivering sufficient CFTR cDNA to correct the Cl[−] transport defect of human airway epithelium derived from individuals with CF. Furthermore, the FIV vectors formulated with EGTA transduced 5–10% of the large airway epithelial cells, a percentage within the range thought to be sufficient to correct the CF defect (2, 3, 7). Finally, the FIV vector corrected the CF defect in vitro for the 6-month life of the epithelial cultures, in vivo infection of tracheal epithelium demonstrated transduction of epithelial cells with progenitor capacity, and the in vivo FIV-transduced epithelia persisted for at least 6 weeks.

Has the battle been won? Hardly — it has only been joined. The FIV vector system is a novel approach to the challenge of transferring CFTR cDNA to the airway epithelium, but there are significant hurdles still to overcome. Like other retroviruses, FIV integrates randomly, making its use in gene therapy a theoretically risky strategy since it could be associated with induction of a malignant phenotype, inability to shut off excess expression of the new gene, and variable expression among cells. The use of VSV-G pseudotyping enables the FIV vector to expand its host range to human cells, but the paucity of receptors for VSV-G on the apical surface airway epithelial cells required Wang et al. (1) to loosen the tight junctions of the airway epithelium to allow the vector to reach the basolateral surface, with its richer density of relevant receptors. Moreover, there is insufficient experience with this technique to determine whether host shut-off of the transferred expression cassette (a problem that has plagued other retrovirus gene transfer vectors) will occur with FIV vectors. Another problem that will have to be overcome is that of developing the methodology to produce large amounts of the modified FIV without contamination by wild-type FIV. FIV is an

immunodeficiency virus that is fatal to domestic cats; if modified to infect humans, there is a small risk that vectors will be contaminated with a recombinant wild-type FIV that is trophic for human cells. Although this scenario appears very unlikely, and although such a virus would probably not have the same growth potential as true wild-type FIV, FIV infection could be devastating for the individual patient and could pose a risk to the environment.

From studies with adenovirus-based vectors, we now know that it is possible to transfer the normal CFTR cDNA to the airway epithelium of individuals with CF at levels that theoretically could prevent the clinical manifestations of the disease (3). The major challenge for CF gene therapy is to get the newly transferred gene to be expressed in a persistent fashion. The study by Wang and colleagues (1) represents an advance toward this goal.

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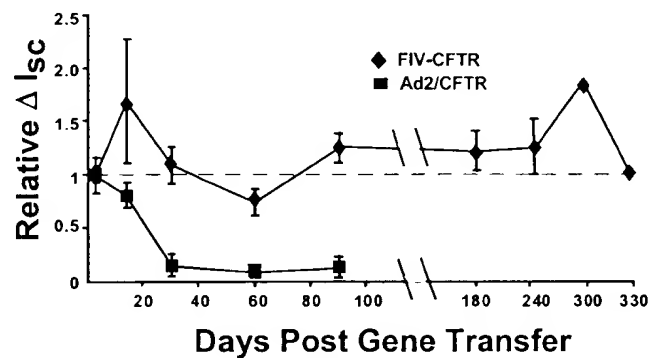


Figure. Persistence of CFTR gene expression in primary cultures of human CF airway epithelia. Cells were transduced with FIV-CFTR by formulating the vector with EGTA and applying it to the apical surface. At the indicated intervals, cAMP-activated Cl channel activity was assessed. Epithelia were studied over a ~12 month period without loss of CFTR gene expression (Wang et al, 1999). CFTR Cl currents gradually declined in cells corrected with adenovirus expressing CFTR. (n=3-4 epithelia/timepoint)

Feline immunodeficiency virus vectors persistently transduce nondividing airway epithelia and correct the cystic fibrosis defect

**Rapid
PUBLICATION**

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Several problems limit the application of gene transfer to correct the cystic fibrosis (CF) Cl⁻ transport defect in airway epithelia. These include inefficient transduction with vectors applied to the apical surface, a low rate of division by airway epithelial cells, failure of transgene expression to persist, and immune responses to vectors or vector-encoded proteins. To address these issues, we used a feline immunodeficiency virus-based (FIV-based) vector. FIV vector formulated with a calcium chelator transduced fully differentiated, nondividing human airway epithelia when applied to the apical surface. FIV-based vector encoding the cystic fibrosis transmembrane conductance regulator cDNA corrected the Cl⁻ transport defect in differentiated CF airway epithelia for the life of the culture (>3 months). When this approach was applied in vivo, FIV vector expressing β -galactosidase transduced 1–14% of adult rabbit airway epithelia. Transduced cells were present in the conducting airways, bronchioles, and alveoli. Importantly, gene expression persisted, and cells with progenitor capacity were targeted. FIV-based lentiviral vectors may be useful for the treatment of genetic lung diseases such as CF.

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Introduction

Gene therapy is the most direct means to correct the Cl⁻ transport defect responsible for cystic fibrosis (CF) lung disease (1–4). However, several problems limit the successful in vivo application of gene transfer to airway epithelia. These include inefficient transduction, immune responses to vectors and vector-encoded proteins, and lack of persistent transgene expression. Such limitations must be overcome if gene transfer is to advance as a treatment for CF and other lung diseases.

Recombinant adeno-associated virus (AAV) (5, 6), Moloney murine leukemia virus (MuLV) (7–11), and lentivirus (12) vectors address the problem of poor persistence due to

their ability to integrate. Lentivirus-based vectors offer the advantage of infecting nondividing cells, a significant consideration in the airways where most cells are mitotically inactive (12). However, limited studies to date suggest that HIV-based lentivirus vectors inefficiently transduce differentiated airway epithelia (13).

A first-generation lentivirus vector derived from the feline immunodeficiency virus (FIV) was recently reported (14). Similar to HIV, FIV vectors transduce nondividing cells (14, 15). Wild-type FIV is antigenically and genetically distinct from HIV and does not infect human cells or cause disease in humans (16). Therefore, FIV-based vectors may offer additional safety fea-

tures compared with HIV-based systems (15). We developed a second-generation FIV vector in which unnecessary *trans*-acting elements (*vif*, *orf2*) were deleted, further reducing the possibility of production of replication competent virus (15). Here, we use this novel FIV-based vector to efficiently transduce airway epithelia in vitro and in vivo. We present novel methods of vector formulation and delivery that facilitate gene transfer to the airways in vivo. FIV vectors may offer advantages over other vectors for airway gene transfer.

Methods

Culture of human airway epithelia. Airway epithelia were isolated from nasal polyps, trachea, and bronchi and grown at the air-liquid interface as described previously (17). All preparations used were well differentiated (>2 weeks old; resistance >1,000 ohm \times cm²) (17, 18). This study was approved by the Institutional Review Board at the University of Iowa.

Drugs and chemicals. Aphidicolin (20 μ g/mL) (Sigma Chemical Co., St. Louis, Missouri, USA) was applied to cells for 24 hours before retroviral transduction to arrest cell growth in G1/S phase (12, 14). To inhibit retroviral RT, 5 μ M 3'-azido-3'-deoxythymidine (AZT; Glaxo Wellcome, Research Triangle Park, North Carolina) was added at the time of viral transduction.

Vector production. The second-generation FIV vector system was reported previously (15). Plasmid constructs

consist of an FIV packaging construct with a deletion in the *env* gene and mutations in *vif* and *orf2*, an FIV vector construct expressing either cytoplasmic *Escherichia coli* β -galactosidase or cystic fibrosis transmembrane conductance regulator (CFTR) genes, and an envelope plasmid in which the human cytomegalovirus (CMV) early gene promoter directs transcription of the vesicular stomatitis virus G protein (VSV-G). In the vector constructs, the CMV promoter directs β -galactosidase expression (FIV- β gal), whereas the MuLV long terminal repeat promoter directs CFTR expression (FIV-CFTR) (19).

VSV-G-pseudotyped FIV vector particles were generated by transient transfection of plasmid DNA into 293T cells as described previously (15). Each FIV- β gal preparation was titrated on NIH 3T3 cells by limiting dilutions; final titers of approximately 5×10^7 to 5×10^9 CFU/mL were obtained. To titer the FIV-CFTR vector, a PCR-based assay system was developed. HT-1080 target cells were transduced with serial dilutions of crude or processed FIV vector preparations in the presence of 4 μ g/mL hexadimethrine bromide (Sigma Chemical Co.). Twenty-four hours later, media were changed and cells were cultured for an additional 24–48 hours. The samples were washed with 1X PBS, and incubated with 2.5 mL of lysis buffer (100 mM Tris [pH 8], 5 mM EDTA, 0.2% SDS, 100 mM NaCl, and 100 μ g/mL pro-

teinase K [QIAGEN Inc., Valencia, California, USA]) at 37°C for 2 hours, and the DNA was precipitated. DNA pellets were washed with 70% ethanol and resuspended in 500 μ L TE buffer, and total genomic DNA was quantified by staining with Hoechst dye H33258 and compared directly against calf thymus DNA standards using the Cyto-Fluor II fluorometer (PerSeptive Biosystems, Framingham, Massachusetts, USA). A total of 100 ng of each genomic DNA sample was subjected to automated PCR (50 μ L volume) using a PE ABI Prism 7700 system (Perkin-Elmer Corp., Norwalk, Connecticut, USA) and a synthetic oligonucleotide primer set directed against FIV packaging signal sequences, yielding an 80-bp product. The resulting fluorescence was detected, and provector copy number titer was expressed as transduction units per milliliter (TU/mL). Titers of 9.7×10^8 to 4.6×10^9 TU/mL were obtained in 2 preparations.

Gene transfer

In vitro. To transduce differentiated human epithelia, the FIV vector was mixed with cell culture medium to a final volume of 100 μ L (~10 moi). This mixture was applied to either the apical surface or the basal surface as described previously (9). To enhance transduction from the apical surface, vector was mixed at a 1:1 (vol/vol) ratio with 12 mM EGTA HEPES/saline solution (pH 7.3) and applied apically for 4 hours as

reported previously for MuLV vectors (9). Polybrene (8 μ g/mL) was included in the transduction solutions.

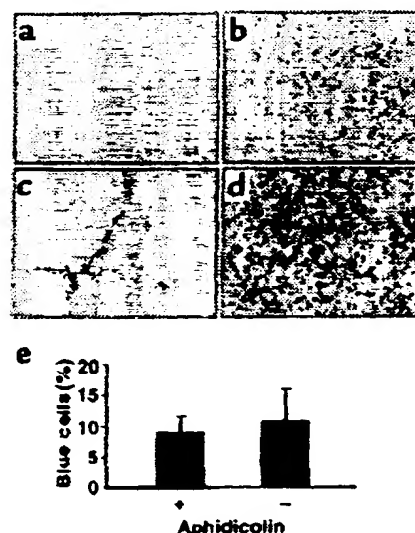
To study the persistence of recombinant FIV-mediated correction of CFTR Cl^- current, results were compared with recombinant adenovirus. We previously reported that adenovirus infects human airway epithelia through the basolateral side by a fiber-dependent mechanism (20). Ad2/CFTR-16 (50 moi) (21) was applied in a 25- μ L volume to the bottom of the epithelia. After 30 minutes, the epithelia were rinsed and returned to the culture dish. Epithelia were studied at intervals for the life of the culture (90–180 days).

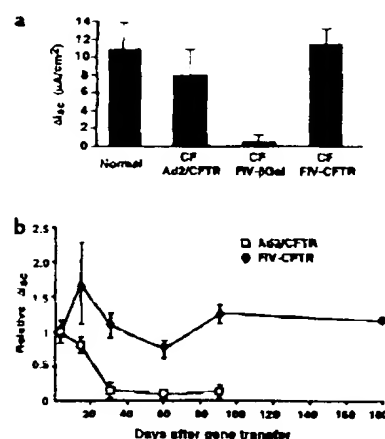
A control experiment was performed to rule out protein transfer or pseudotransduction as reported for concentrated AAV and retroviral vectors (22, 23). When applying the FIV- β gal vector/EGTA solution to the apical surface, cells were treated with AZT to inhibit the retroviral RT. AZT-treated cells showed no significant expression of vector-encoded product (not shown), confirming that FIV-vector transduction under these experimental conditions was not due to protein transfer.

In vivo. For tracheal gene transfer, adult New Zealand white rabbits were anesthetized with 32 mg/kg ketamine, 51 mg/kg xylazine, and 0.8 mg/kg acepromazine intramuscularly; a ventral midline incision was made; and a tracheotomy was performed. An approximately 1.5-cm tracheal segment cepha-

Figure 1

FIV vectors transduce nondividing airway epithelia *in vitro*. (a) Application of FIV- β gal (moi 10) to the apical surface of the epithelial sheet resulted in no gene transfer. Representative en face view of X-gal stained epithelia 3 days after vector application. (b) FIV- β gal vector applied to airway epithelia from the basolateral surface *in vitro* (moi ~10) resulted in gene transfer. Representative en face view is shown. (c) Gene transfer from the apical surface with VSV-G FIV is enhanced by physical disruption of epithelia. The epithelial sheet was scratched with a pipette tip prior to apical vector application. En face view shows gene transfer (gray cells) only along the area of epithelial disruption. (d) FIV transduces aphidicolin growth-arrested cells (en face view). Vector was applied to apical surface in the presence of 6 mM EGTA in hypotonic buffer. Approximately 17% of epithelia were transgene positive (range 12–22%; $n = 5$ epithelia from 2 different preparations). For cells in control media without aphidicolin, approximately 20% of cells expressed the transgene (data not shown; range 8–30%; $n = 5$ epithelia from 3 different preparations). (e) Quantification of gene transfer results under conditions shown in d (mean \pm SEM; $n = 5$ epithelia; 3 different preparations).



**Figure 2**

FIV-CFTR corrects the CF Cl^- transport defect. CF epithelia were transduced from the apical surface in the presence of EGTA with ~10 moi of FIV vector expressing either β -galactosidase or human CFTR. For comparison, another group of CF cells received Ad2/CFTR (moi 50, basolateral application). At time points of 3, 13, 30, 60, 90, and 180 days later, epithelia were mounted in Ussing chambers, and the change in short circuit current was measured in response to cAMP agonists ($\Delta \text{Isc}_{(\text{IBMX}/\text{Forsk})}$). (a) Comparison of $\Delta \text{Isc}_{(\text{IBMX}/\text{Forsk})}$ in response to cAMP agonists in CF epithelia transduced with adenovirus or FIV vectors, 3 days after gene transfer. Both Ad- and FIV-transduced epithelia express cAMP-activated Cl^- currents similar to normal cells ($n = 5$ CF epithelia; $n = 5$ normal epithelia, for each time point). (b) CFTR expression persists in FIV-transduced epithelia. CF epithelia were transduced with FIV- β gal, FIV CFTR, or Ad2/CFTR and $\Delta \text{Isc}_{(\text{IBMX}/\text{Forsk})}$ measured at the indicated time points after gene transfer ($n = 5$ CF epithelia; $n = 5$ normal epithelia, for each time point). Data from each experiment were normalized to the mean $\text{Isc}_{(\text{IBMX}/\text{Forsk})}$ seen 3 days after infection. One CF preparation was viable 6 months after gene transfer.

lad to the tracheotomy was isolated and cannulated on each end with PE 330 tubing (Becton Dickinson, Parsippany, New Jersey, USA). The tracheal segment was rinsed and then filled with a solution of 12 mM EGTA in 10 mM HEPES buffer (pH 7.4, "hypotonic buffer") for 30–60 minutes. The EGTA solution was then replaced with 300 μL of FIV- β gal vector (titer 1×10^8 to 5×10^8 CFU/mL). The vector solution was left in place for 45 minutes, and then the cannulae were removed and the incisions closed. Five days or 6 weeks later, the tissues studied for β -galactosidase expression. For lower airway gene transfer, a PE50 catheter passed via the trachea until it lodged in a subsegmental bronchus. A total of 200–600 μL of FIV- β gal formulated in hypotonic buffer with 6 mM EGTA was instilled. Five days later, the tissues were studied for β -galactosidase expression.

Tissue histochemistry

β -galactosidase expression. Epithelial cells were fixed and X-gal stained as reported elsewhere (9, 24). Epithelia counterstained with DAPI were examined microscopically en face for β -galactosidase expression. The percentage of β -galactosidase-positive cells was determined by counting a minimum of 1,000 cells from representative en face views of each treated epithelia.

Rabbit tissues were fixed in 2% paraformaldehyde/PBS overnight, X-gal stained, and embedded in paraffin, and sections were cut for histological examination (24). Sections were counterstained with nuclear fast red or hema-

toxylin and eosin. To determine the percentage of β -galactosidase-expressing cells in each 1.5- to 2-cm tracheal specimen, serial sections were cut every 20 μm , and ≥ 50 slides were examined. To quantify gene transfer to lower airways, the blue tissue areas of the X-gal-stained lungs (see Figure 4a) were dissected and embedded in paraffin, and serial sections cut at 40- μm intervals. The percentage of β -galactosidase-positive cells in lower airway tissues was quantified by cell counting. β -galactosidase-expressing cells were categorized by the size of the airway in which expression was noted (>750 μm , 500–750 μm , 250–500 μm , 0–250 μm) using a calibrated eyepiece reticle. To identify the cell types expressing β -galactosidase, standard morphologic criteria were used. Transgene-expressing cells were identified by their physical characteristics: (a) ciliated cells are columnar cells with cilia; (b) goblet cells are columnar cells containing secretory granules; (c) basal cells are basally located cuboidal cells having no contact with the mucosal surface; (d) intermediate cells are columnar cells in the lower half of the epithelium having no contact with the lumen; (e) Clara cells are nonciliated, columnar to cuboidal surface cells that are more prevalent in the distal airways; and (f) alveolar type II cells are cuboidal, "corner" cells of the alveolar epithelium.

Measurement of transepithelial CFTR Cl^- current. To measure transepithelial bioelectric properties, epithelia were mounted in Ussing chambers and studied 3, 13, 30, 60, 90, and 180 days

after gene transfer as described previously (17). The cAMP-stimulated Isc ($\text{Isc}_{(\text{IBMX}/\text{Forsk})}$) is the increase in current after basolateral addition of cAMP agonists (10 μM forskolin plus 100 μM 3-isobutyl 1-methylxanthine [IBMX]) in the presence of 10 μM amiloride. Data from each experiment were normalized to the mean $\text{Isc}_{(\text{IBMX}/\text{Forsk})}$ seen 3 days after transduction. CF airway epithelia were genotyped and were compound heterozygotes for the ΔF508 mutation ($\Delta\text{F508}/-$, $\Delta\text{F508}/1717-16\text{-A}$). 1717-16-→A

Results

FIV vectors transduce nondividing airway epithelia *in vitro*. On the basis of previous literature (25, 26) as well as our own studies with MuLV (9, 11), we suspected that the receptors for VSV-G-pseudotyped FIV vectors were only expressed on the basolateral surface of airway epithelia. Indeed, when VSV-G-pseudotyped FIV- β gal was applied to the apical surface, no gene transfer occurred (Figure 1a). In contrast, FIV- β gal applied to the basolateral surface transduced the epithelia (Figure 1b). We hypothesized that if epithelial junctions were opened, FIV vector particles would have a better chance to interact with cell-surface receptors and gain entry when applied apically. Scratching the epithelial sheet with a pipette tip before applying vector to the apical surface enhanced gene transfer only in areas where the cells were mechanically disrupted (Figure 1c). Thus, if receptors were made accessible, gene transfer was achieved with VSV-G-pseudotyped FIV vectors.

To demonstrate that FIV vectors

transduce nondividing epithelia, we performed experiments in the presence or absence of aphidicolin-induced growth arrest (12, 14). As we found previously that calcium chelation with EGTA and hypotonic solutions disrupted epithelial junctions and facilitated gene transfer with apically applied MuLV vectors (9), a similar approach was used with the FIV vector. Formulation of FIV- β gal with 6 mM EGTA in a hypotonic buffer (~10 mO) greatly increased gene transfer from the apical surface (Figure 1d). Approximately 17% of epithelia growth-arrested with aphidicolin were transgene positive 3 days after transduction, whereas approximately 20% of epithelia in control media were transduced (Figure 1, d and e). Previous studies showed that approximately 7% of cells are proliferating in this model as assayed by BrdU histochemistry (9). The EGTA solution alone had no effect on cell proliferation as assayed by BrdU histochemistry (data not shown). Thus, when allowed access to receptors, FIV vectors effectively transduced nondividing epithelia.

FIV vectors coding for CFTR persistently correct the CF Cl^- transport defect. On the basis of the results in normal human airway epithelia, we hypothesized that recombinant FIV vectors expressing CFTR would complement the Cl^- transport defect in well-differentiated CF epithelia. For these studies, we used primary organotypic cultures of human airway epithelia from patients with CF, for the following reasons. Primary cultures of differentiated human CF airway epithelia recapitulate several important aspects of in vivo airway epithelial biology and CF disease. Cells cultured in this fashion morphologically resemble the human airways in

vivo (17, 18). Similar to the in vivo human airways, they are relatively resistant to transduction by both viral and nonviral vectors applied to the apical surface (9, 17, 27, 28). They manifest the electrolyte and liquid transport defects characteristic of CF (18, 29). Importantly, unlike the long-term survival and minimal evidence of lung disease reported for CFTR-null mice or mice with specific human CFTR mutations (30–35), cultured human CF epithelia show an increased susceptibility to bacterial infection (36).

Tracheal epithelia were transduced in vitro from the apical surface with FIV-CFTR. Correction of the CFTR Cl^- transport defect was assayed by measuring the change in short-circuit current in response to cAMP agonists ($\Delta \text{Isc}_{(\text{IBMX/Forisk})}$) from 3 days to 6 months after gene transfer (Figure 2). CF epithelia transduced with FIV-CFTR or adenovirus expressing CFTR uniformly demonstrated Cl^- secretion in response to cAMP agonists, whereas control cells treated with FIV- β gal showed no response (Figure 2a). The Cl^- secretory responses in the corrected cells (Figure 2, a and b) were similar to those measured in normal airway epithelia (Fig-

ure 2, a and b). In cells transduced with adenovirus, $\Delta \text{Isc}_{(\text{IBMX/Forisk})}$ gradually declined over time. In contrast, the net $\Delta \text{Isc}_{(\text{IBMX/Forisk})}$ in FIV-transduced cells remained stable (Figure 2b). In 1 FIV-CFTR-transduced culture that remained viable for 6 months, cAMP-activated Cl^- current persisted at similar levels as day 3 (Figure 2b).

FIV vectors transduce airway epithelia in vivo. FIV vectors might also effectively transduce airway epithelia in vivo if VSV-G receptors were accessible. We used a similar protocol of epithelial junction disruption to test FIV vectors in vivo. After EGTA pretreatment, FIV- β gal vector was applied to the luminal surface of the trachea in adult rabbits. Five days later, the tissues were removed and studied for β -galactosidase expression. As shown in Figure 3, a–e, cells throughout the epithelium expressed the transgene. The transduction efficiency was $4.8 \pm 5.6\%$ (mean \pm SEM; range: 1–14%; $n = 4$). The treated epithelia appeared intact, without evidence of injury or inflammatory cell infiltrates. Of note, basal cells, intermediate cells, and both ciliated and nonciliated surface cells expressed β -galactosidase (Figure 3, b–e). Previous studies in several species

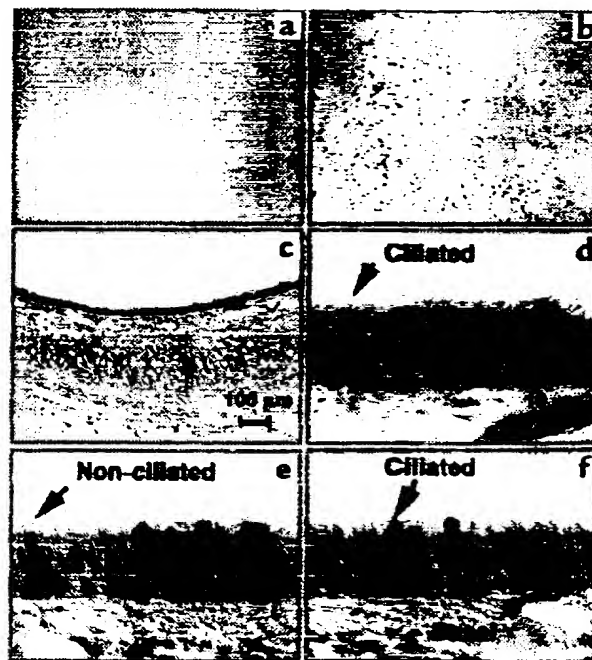
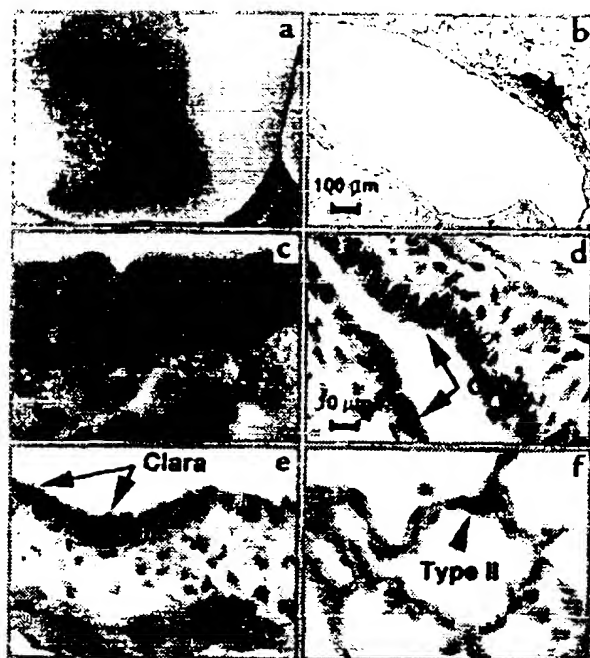
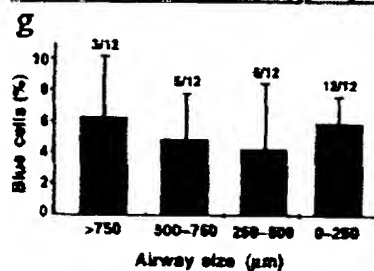


Figure 3

Gene transfer to rabbit tracheal epithelia in vivo using FIV- β gal vector. Panels show results 5 days after gene transfer. Low magnification en face view of X-gal-stained trachea from control (a) or FIV vector-treated trachea (b). Blue cells were only seen in the trachea transduced with the FIV vector (b). (c) Low-magnification view of X-gal-stained tracheal section. β -galactosidase-expressing cells are noted at both the surface and basal cell levels of the transduced epithelium. (d–f) Higher-magnification views of tracheal epithelium showing cell types expressing β -galactosidase. No inflammatory cells were noted in control or transduced specimens ($n = 4$ animals). Scale bar in d also applies to e and f.

**Figure 4**

FIV- β gal transduction of lower airway epithelia 5 days after gene transfer. (a) En face view of pleural surface of lung after fixation and X-gal staining showing β -galactosidase-expressing cells. All treated animals had similar segments of β -galactosidase-expressing cells extending to the pleural surface. (b-f) Higher magnification views of tissue sections showing lower airway and parenchymal cells transduced. (b) Low-magnification view of a large bronchus ($>750 \mu\text{m}$ diameter) demonstrating patches of β -galactosidase-expressing cells extending around the circumference of the epithelium. (c) High-magnification view of expression in a large bronchus ($>750 \mu\text{m}$ diameter) showing expression in ciliated cells and basal cells. (d) High-magnification view of expression in a medium sized airway ($500\text{--}750 \mu\text{m}$ diameter) demonstrating expression in nonciliated surface cells (Clara cells). (e) β -galactosidase expression in a small bronchus ($250\text{--}500 \mu\text{m}$ diameter) showing expression in nonciliated surface cells (Clara cells). (f) Distal lung sample (airways $0\text{--}250 \mu\text{m}$ diameter) showing expression in cuboidal cells consistent with alveolar type II cells. (g) Gene transfer expressed as a function of airway size. Numbers above each bar represent the number of animals with transduced cells in the corresponding region. Tissues from 12 animals were studied. Scale bar in d also applies to e and f.



epithelia expressing β -galactosidase were noted throughout the segment where virus was instilled, from cartilaginous bronchi with diameters greater than $750 \mu\text{m}$ out to the alveoli (Figure 4, b-e). The percentage of transgene-expressing cells across all airway sizes was $4.9 \pm 2.2\%$ (mean \pm SEM; range: 2.6-10.3%; $n = 12$). β -galactosidase expression occurred more frequently in the smaller airways than the larger airways, as might be expected with the method of vector introduction (Figure 4g). Importantly, the morphology of the transduced airway epithelia appeared normal, and all cell types of the lower airways were transduced, including proposed progenitors such as basal cells, nonciliated surface cells (Clara cells), and alveolar type II cells. Vector instillation without the EGTA formulation resulted in no significant gene transfer (not shown).

FIV gene transfer to airway epithelia persists in vivo. Animals treated with FIV- β gal vector intratracheally were evaluated 6 weeks after gene transfer for persistence of gene expression. In contrast to the results at day 5 (Figure 3, a-e), larger clusters of β -galactosidase-positive cells were noted on both the en face views and the cross sections of the trachea (Figure 5), suggesting

that targeted cells clonally expanded over time. As shown in Figure 5, we noted β -galactosidase-expressing cells throughout the epithelium. The percentage of β -galactosidase-expressing cells was $2.5 \pm 2\%$ (mean \pm SEM; range: 0.4-5.4%; $n = 4$). When compared with the level of expression at 5 days (Figure 3), this change was not significant ($P = 0.5$ by t test). Transduced cell types included basal cells, nonciliated surface cells, ciliated surface cells, cells containing mucus granules, intermediate cells, and rarely, epithelia of submucosal glands. As noted at the 5-day time point, the epithelial morphology appeared normal.

Discussion

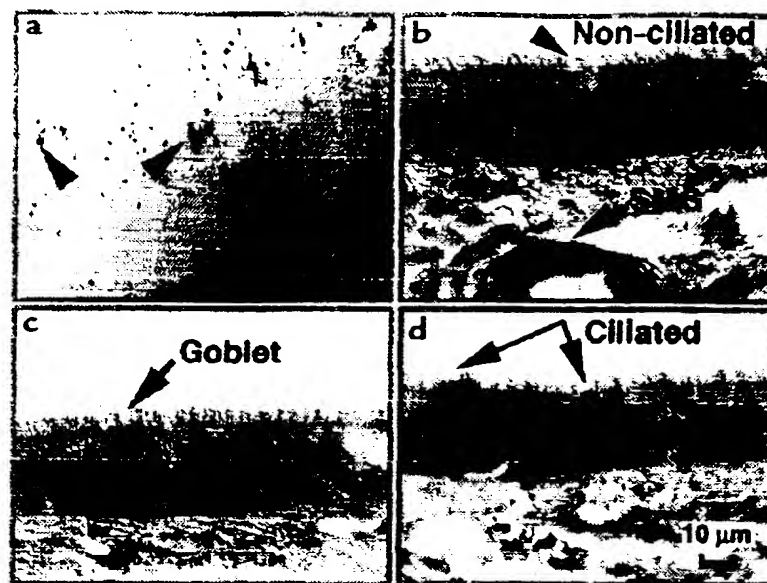
For CF lung disease to be treated by gene therapy, there must be lasting correction of defective Cl^- transport. In these studies, we make significant progress in addressing several fundamental limitations for gene transfer to airway epithelia. A shortcoming of most current vectors is their inability to effectively transduce airway epithelia when applied to the apical surface. The native receptors for many recombinant viruses are distributed on the basolateral cell surface of polarized cells. Previous investigators noted that VSV-G pseudotyped HIV lentivirus

showed that the mitotic labeling indices for cells other than basal cells are very low ($<1\%$) in adult airway epithelia (37). While these data suggest that FIV vectors transduce both dividing and nondividing airway cells in vivo, we cannot rule out the possibility that EGTA/hypotonic treatment stimulated some stationary-phase cells to divide. In the absence of EGTA formulation, there was no gene transfer (data not shown).

CF lung disease begins in the small airways. To target the intrapulmonary airways, a small catheter was passed transtracheally into the peripheral airways, and hypotonic/EGTA formulated FIV- β gal vector was instilled. This approach allowed the vector solution to be concentrated within a relatively small area. As shown in Figure 4, a-e, we uniformly noted focal areas of gene transfer in the lung 5 days later. When serial sections of tissue were studied,

Figure 5

β -galactosidase expression persists in vivo 6 weeks after gene transfer. (a) En face view demonstrating β -galactosidase-positive cells in the trachea. Larger clusters of blue cells (arrows) were noted at 6 weeks than at the earlier time point, suggesting clonal expansion of transduced cells. (b-d) Cross sections of tissue shown in a. Multiple cell types were targeted as indicated by the arrows. Clusters of β -galactosidase-positive cells were noted, suggesting clonal expansion of targeted cells ($n = 4$ animals). Epithelial morphology appeared normal as determined by examination of hematoxylin and eosin stained sections. Scale bar in d also applies to b and c. SMG = submucosal gland.



(13) and MuLV (10) inefficiently transduced differentiated airway epithelia in vivo. We (9, 11) and others (38) reported this limitation with MuLV retrovirus envelopes, and there is precedence for such a polarity of gene transfer to airway epithelia with other delivery systems including AAV (28, 39) and adenovirus vectors (20, 40) as well as cationic lipids (41). Although VSV-G-pseudotyped FIV transduced cells poorly from the apical surface, this limitation was overcome using hypotonic/EGTA vector formulation to transiently open epithelial junctions (42-44). Under these conditions, cells throughout the epithelium were transduced in vitro and in vivo, and the CFTR Cl⁻ transport defect was corrected in vitro.

The transduction efficiency of FIV vectors formulated with EGTA is within the range of 6-10% believed sufficient to correct the CF defect (45). Although this work focused on a single vector dose administration, it is possible that lentiviral vectors may be readministered with minimal immune responses and further increase the number of permanently corrected cells (46). This vector formulation method represents a technical advance for vector administration to polarized epithelia in vivo. To translate such a result to patients, epithelial junctions in the human lung could be transient-

ly opened pharmacologically to facilitate vector access to receptors. Aerosol studies in humans show that it is technically feasible to transiently expose the airway epithelium to hypotonic conditions (H₂O aerosol) (47) or calcium chelators (EDTA aerosol) (48). In addition, pulmonary lavage under anesthesia might be developed to deliver vector solutions to the human airways. Such whole lung lavage procedures are currently used clinically to treat patients with alveolar proteinosis (49). With current integrating vectors, a lavage approach may be required to facilitate access to receptors on airway progenitor cells, as some of these cell types reside below the mucosal surface (e.g., basal cells, intermediate cells) (50-54).

A further encouraging result of this work was the normal morphology of the airway tissues after gene transfer. There was no evidence of cellular infiltration with immune effector cells when the rabbit airways were examined at the level of light microscopy (Figures 3, 4, and 5). Although this does not eliminate the possibility of any immune response, it contrasts with the cellular responses noted with adenoviral vectors (24, 55-57). Studies with HIV-based lentiviral vectors to date show no evidence of cellular immune responses at the sites of administration in vivo (46). Further-

more, HIV-based vectors (46) and MuLV vectors (58) can be administered a second time in vivo, suggesting that humoral immune responses may not prevent repeated dosing. Thus, enveloped viruses such as lentivirus or MuLV may be less immunogenic when delivered to the lung.

FIV vectors corrected the CFTR defect in vitro for the life of the culture. The failure of adenoviral-mediated CFTR correction to persist reflects both the lack of integration and the gradual loss and dilution of expressing cells by cell division. The persistence of CFTR correction in the FIV vector-transduced cells indicates successful targeting of cells with progenitor capacity. FIV-transduced epithelia also persisted in the trachea in vivo. There are several possible explanations for the small decline in gene expression we noted in the trachea between 5 days and 6 weeks. These include immune responses to the transgene, transcriptional shut off of the CMV promoter, loss of terminally differentiated cells that are targeted (i.e., ciliated cells), and variability related to the effective moi achieved in each animal. Future studies will evaluate these possibilities.

To our knowledge, this is the first evidence of in vivo transduction of airway epithelia with a lentiviral vector. As reported with HIV-based lentiviral vectors (12, 13, 46) or equine infec-

tious anemia virus (EIAV) vectors (59), the present studies show that recombinant FIV vectors transduce nondividing cells in vitro. This is an important finding, as the majority of airway epithelia are mitotically quiescent in the postnatal airways (60-62). FIV vectors transduced epithelial cells throughout the adult rabbit airways in vivo, and gene expression persisted for 6 weeks. Further studies are needed to document the proliferation status of cells at the time of transduction in vivo. Cell types believed to have progenitor capacity in the airway epithelium such as basal cells, intermediate cells, and nonciliated surface cells were transduced (50-52, 54). Integration of the proviral DNA into the host cell chromosome should allow the persistent expression of a therapeutic gene such as CFTR. These studies provide a strong rationale for a vector delivery approach that may be used for the treatment of genetic lung diseases.

Acknowledgments

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In vivo adenovirus-mediated gene transfer to lungs via pulmonary artery

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Lemarchand, Patricia, Michael Jones, Claire Daniel, Izumi Yamada, Andrea Mastrangeli, and Ronald G. Crystal. In vivo adenovirus-mediated gene transfer to lungs via pulmonary artery. *J. Appl. Physiol.* 76(6): 2840-2845, 1994. —On the basis of the knowledge that the pulmonary and bronchial circulations have extensive anastomoses, we hypothesized that gene transfer to the endothelium of both pulmonary and bronchial circulations might be achieved with replication-deficient recombinant adenovirus (Ad) vectors administered to the pulmonary circulation. To evaluate this concept, the right upper lobe branches of the sheep pulmonary artery and vein were temporarily occluded and a replication-deficient recombinant Ad vector containing the *Escherichia coli lacZ* reporter gene coding for β -galactosidase (β -Gal) was infused into the lumen of the occluded pulmonary artery. After 15 min, the pulmonary circulation was restored, and 1 or 4 days later the lungs were evaluated by histochemical analysis for β -Gal activity. Gene transfer and expression were positive in 13 of 17 evaluated sheep. No β -Gal activity was detected in any category of cells of uninfected lobes. As hypothesized, β -Gal activity was detected in endothelial cells of the right upper lobe pulmonary and bronchial circulations. Unexpectedly, gene transfer was also observed in epithelial cells of the alveoli and the airways (bronchi and bronchioles) as well as in the epithelium of submucosal glands. These studies demonstrate that it is possible to use Ad vectors for transfer and expression of genes to lung parenchymal cells served by both the pulmonary and bronchial circulations. Furthermore, whereas administration of such vectors via the airways results in gene transfer only to the epithelium, pulmonary artery administration permits gene transfer to both endothelium and epithelium, thus expanding the target range of Ad gene transfer to the lungs.

cystic fibrosis; endothelium; epithelium; pulmonary circulation

IN VIVO GENE THERAPY is a therapeutic strategy in which genetic material is transferred directly to cells within the individual to be treated in a fashion that permits the genetic material, either directly or through its products, to be of therapeutic benefit (7). Among the various strategies developed for in vivo gene therapy, recombinant adenovirus (Ad) vectors have several features that make them suitable for gene therapy to the lung (5, 16, 26, 28, 29, 34), and studies with experimental animals have demonstrated that when Ad vectors are administered to the lungs via the air route there is efficient transfer and expression of the exogenous gene in the airway epithelium

(25, 28, 29). However, the available evidence suggests that the air route administration of Ad vectors is not effective to transfer genes to the endothelium of the lung vasculature. In contrast, Ad vectors administered directly into the lumen of large vessels are capable of transferring genes to the endothelium of both arteries and veins (22). Thus, it should be possible to achieve gene transfer to the pulmonary vascular endothelium if the Ad vectors are administered via the pulmonary circulation such that the vectors could come in direct contact with the endothelium. Theoretically, such an approach may also allow access of the vector (and hence gene transfer) to the bronchial endothelium. In this regard, anastomoses of the pulmonary and bronchial circulations are present in the lungs of humans and many other species at both the arteriolar and capillary levels (27, 32).

To evaluate this concept, a replication-deficient recombinant Ad vector containing the *Escherichia coli lacZ* reporter gene coding for β -galactosidase (β -Gal) was infused into the lumen of the right upper lobe (RUL) pulmonary artery. This was carried out in sheep, an experimental animal with pulmonary and bronchial circulations similar to those in humans (8, 23). The data demonstrated that with this approach Ad vectors can transfer genes to the endothelium of the pulmonary and bronchial circulations. Unexpectedly, the data also demonstrated that administration of an Ad vector used in this fashion permits gene transfer to the alveolar epithelium, the bronchial epithelium, and the epithelium of the submucosal glands.

METHODS

Ad vector. The Ad vector Ad.RSV β gal is a replication-deficient recombinant Ad based on Ad type 5 in which the majority of the E1 and E3 regions have been deleted and that contains the Rous sarcoma virus promoter followed by the SV40 nuclear localization signal and the *E. coli lacZ* reporter gene encoding for β -Gal (25, 33). β -Gal is an intracellular protein that can be detected with a convenient in situ histochemical assay, permitting detection of successful gene transfer and expression as evidenced by a blue color of the cell expressing β -Gal (11). For details concerning the general production, purification, and evaluation of Ad.RSV β gal, see Refs. 21, 22, 25, 30, and 33.

In vivo gene transfer via the pulmonary artery. The study was carried out with female sheep weighing 30–35 kg (mixed Rambouillet, Dorset, Suffolk breed). The administration of Ad

vectors was performed under general anesthesia and sterile surgical conditions. Anesthesia was induced with intravenous pentobarbital sodium (25 mg/kg) and diazepam (10 mg) and was maintained with 1-2% isoflurane with oxygen via an endotracheal tube and a volume-cycled ventilator. A right posterolateral thoracotomy was performed, and the pulmonary vein from RUL and the two branches of the pulmonary artery to RUL were dissected and encircled with Silastic tourniquets. Bovine lung heparin sodium (300 U/kg) was injected intravenously for systemic anticoagulation while the vessels were occluded. Preliminary studies were carried out in three sheep with infusion of the Ad vector into the pulmonary artery without vessel occlusion. After analysis showed some delivery to lung cells in the distribution of the pulmonary artery but no gene transfer to the endothelium of the bronchial circulation (see RESULTS), all subsequent animals were studied using occlusion of the pulmonary artery and vein. After the occlusion of both RUL pulmonary artery and vein, the Ad vector ($1-3 \times 10^{11}$ plaque-forming units in 8 ml of 0.9% saline) was infused into the lumen of the cranial segmental branch of the right apical lobe pulmonary artery by using a 3-Fr polypropylene catheter. The catheter was removed, and the puncture site was repaired. After 15 min of incubation (a time determined by previous studies of *in vivo* gene transfer to the endothelium of large vessels; Ref. 21), the tourniquets were released and circulation through RUL was restored. The thoracotomy incision was closed, and the animal was allowed to recover. Prophylactic antibiotics, gentamicin (3 mg/kg) and penicillin G (9×10^5 U), were administered twice daily for 3 days. Systemic anticoagulation with heparin was initially maintained for several days after surgery; however, after the death of three animals from hemorrhage, systemic anticoagulation was discontinued and all subsequent animals received only a single dose of heparin just before the occlusions of RUL pulmonary artery and vein. All animals were killed 1 or 4 days after Ad administration. The lungs were inflated and excised en bloc.

Evaluation of gene transfer and expression. To evaluate *in vivo* gene transfer and expression, the lungs were fixed (2 h, 4°C) in 2.0% formaldehyde and 0.2% glutaraldehyde. The presence of the *lacZ* gene product, β -Gal, was determined by staining the lungs with the X-Gal reagent (22, 25) for 6 h. After staining, samples were postfixed in the same fixative, embedded in paraffin, cut into 5- μ m sections, and counterstained with nuclear fast red or periodic acid-Schiff. Cells were considered positive for expression of the *lacZ* gene when a nuclear-dominant blue color was observed. For each animal the left lung (which was not exposed to the Ad vector) was used as control.

RESULTS

Preliminary experiments ($n = 3$ sheep) with injection of the recombinant Ad vector in the pulmonary artery without vessel occlusion led to gene transfer to the endothelium of the pulmonary arteries, pulmonary capillaries, and alveolar epithelium but not to the endothelium of the bronchial circulation and airway epithelium.

Gene transfer was attempted with temporary occlusion of both pulmonary artery and vein in a total of 20 sheep. Three sheep died during surgery of hypotension with peripheral vasodilation; gene transfer was not evaluated, and these animals were eliminated from the study. Three other sheep, all under systemic anticoagulation after surgery, died at days 1 ($n = 2$) and 3 ($n = 1$) of hemorrhage; these animals were included in the evaluation of gene transfer. In a total of 17 sheep evaluated for

TABLE 1. Evidence of β -Gal activity in various lung cells of sheep after adenovirus-mediated gene transfer

Lung Parenchymal Cells	Positive β -Gal Activity Observed	
	RUL	Left lung (control)
Endothelium		
Pulmonary arterial	13/13	0/13
Pulmonary capillary	10/13	0/13
Pulmonary venous	8/13	0/13
Bronchial capillary	8/13	0/13
Epithelium		
Alveolar	10/13	0/13
Bronchial	8/13	0/13
Bronchiolar	8/13	0/13
Submucosal gland	6/13	0/13
Smooth muscle	0/13	0/13
Fibroblasts	0/13	0/13
Cartilage	0/13	0/13

Data were derived from animals in which successful gene transfer was observed (13 of 17 evaluated sheep; see text for details. β -Gal, β -galactosidase, RUL, right upper lobe.

gene transfer, X-Gal staining revealed β -Gal activity in the sections of RUL in 13 of 17 animals. In the remaining four sheep, X-Gal staining revealed no β -Gal activity in the sections of RUL evaluated. The following description of gene transfer reflects data from these 13 animals only (Table 1). In the 13 animals where β -Gal activity was detected in RUL, no β -Gal activity was detected after X-Gal staining in any sections evaluated in the left lung (a region not exposed to the Ad vector and thus used as control). Evaluation of the endothelium of RUL pulmonary circulation revealed transfer and expression of β -Gal (Fig. 1). In the control left lung, no β -Gal activity was observed in the endothelium (Fig. 1A). In contrast, after administration of Ad.RSV β gal in RUL pulmonary artery, X-Gal staining showed extensive β -Gal expression in the endothelium of the pulmonary arteries and/or arterioles (Fig. 1B). β -Gal expression was limited to the endothelial layer in the vessels. There was also β -Gal expression in the endothelium of pulmonary veins (Fig. 1C) and pulmonary capillaries (Fig. 1D).

Examination of the epithelial cells of the lungs showed no β -Gal activity in the left lung (Fig. 2A). In contrast, β -Gal activity was observed in the bronchiolar epithelium of RUL and in the endothelium of the pulmonary arteries and the capillaries adjacent to the bronchioles (Fig. 2B). β -Gal activity was also detected in the epithelium of RUL proximal bronchi (Fig. 2C), and evaluation by high magnification demonstrated that all categories of cells, including ciliated cells, undifferentiated cells, as well as basal cells, expressed β -Gal activity (data not shown). β -Gal activity was also detected in the epithelium of the submucosal glands of RUL bronchi (Fig. 2D), the canals (Fig. 2E), and within the adjacent bronchial capillaries (Fig. 2D). Examination of the alveolar epithelium showed β -Gal activity in RUL alveoli predominantly in type 2 epithelial cells but also in some type 1 cells (Fig. 2F).

Although the exogenous gene was transferred and expressed in the endothelium of the pulmonary arteries in each animal, gene transfer and expression were patchy and variable in other lung structures (Table 1).

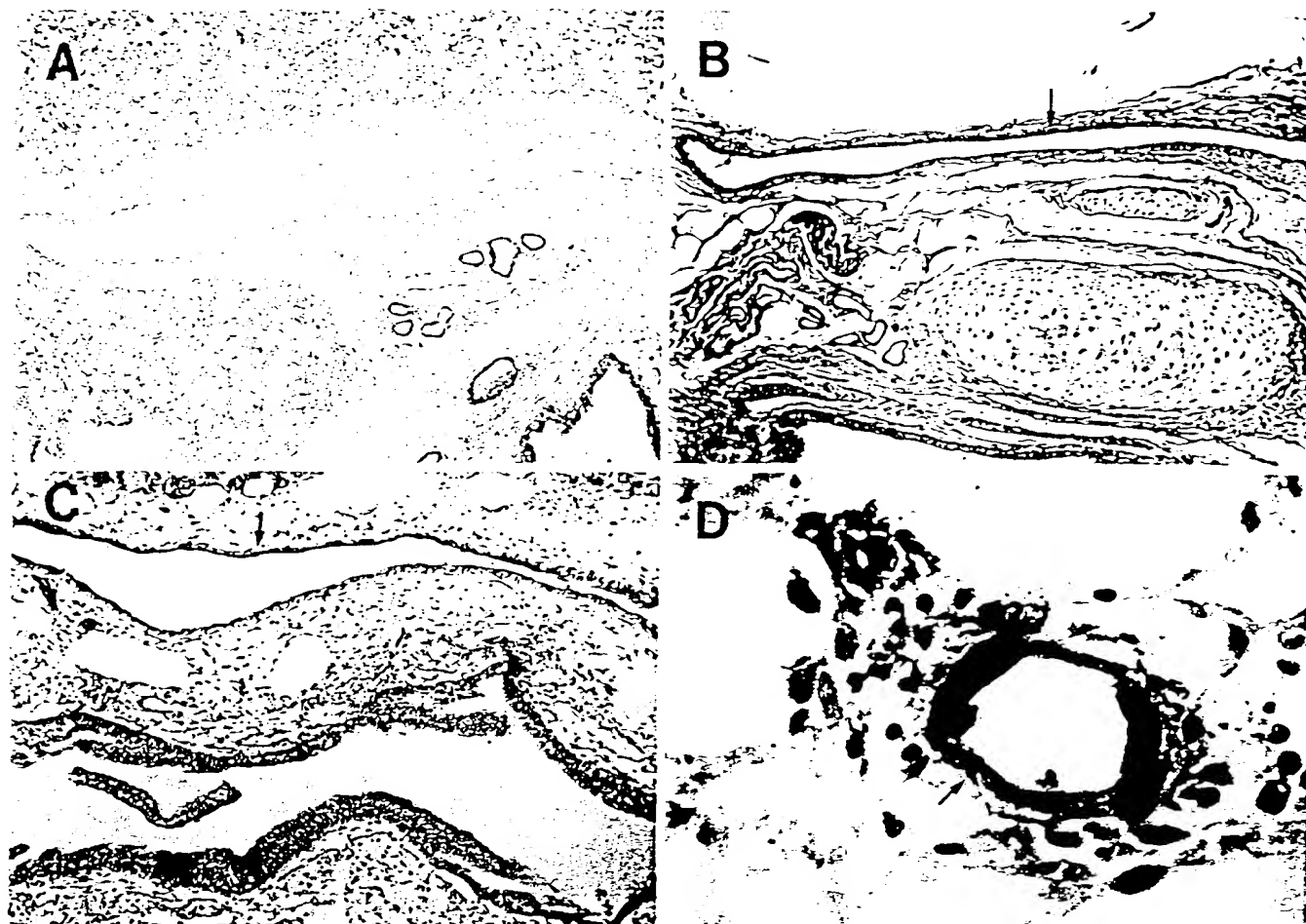


FIG. 1. Adenovirus (Ad)-mediated transfer and expression of *lacZ* gene in pulmonary vasculature after in vivo pulmonary artery administration of vector. After occlusions of right upper lobe (RUL) artery and vein of adult sheep, replication-deficient recombinant Ad vector containing *lacZ* reporter gene coding for β -galactosidase (β -Gal) (Ad.RSV β gal) was infused into cranial branch of RUL pulmonary artery. After 15 min, pulmonary circulation was restored. Lungs were evaluated by histochemical analysis for β -Gal activity (blue) after 1 (A and B) or 4 days (C and D). Shown are photographs of paraffin sections counterstained with nuclear fast red; β -Gal activity (blue) is present in endothelium of vessels of pulmonary circulation. Arrows, β -Gal activity in endothelium of pulmonary artery (B), pulmonary vein (C), and pulmonary capillaries (D). A: left lung, uninfected. B-D: RUL, Ad.RSV β gal infected. A-C, $\times 100$; D, $\times 400$.

DISCUSSION

The present study demonstrates that, by using the pulmonary arterial circulation with transient occlusion of the pulmonary artery and vein, it is feasible with an Ad vector to transfer genes to the lungs in vivo. Furthermore, the exogenous gene transferred in this fashion is expressed in all categories of endothelium and epithelium of the lung, including the endothelium of the pulmonary and bronchial circulations; epithelium of alveoli, bronchioles, and bronchi; and submucosal glands. Several other techniques have been used to transfer genes to the lungs in vivo, including liposomes (17, 36) and Ad-polysyn complexes (14); however, none of these studies showed gene transfer and expression in both airway epithelial and endothelial cells of the bronchial circulation.

In our study, 4 of 17 sheep did not show any gene transfer and expression. One possible explanation is that these animals presented antibodies against Ad type 5 before administration of the virus. It is conceivable that the

lack of expression in these four animals resulted from anti-Ad immunity, but although this was not evaluated in the present study, it would be very unusual for experimental animals to have anti-human Ad antibodies *de novo*.

The anatomy of the systemic vasculature of the ovine lung has been carefully documented (2, 3, 8-10). Sheep have multiple sources of systemic arterial blood flow to the lung that anastomose with each other as well as with the pulmonary circulation (9, 10). When injected into the pulmonary artery, the recombinant Ad vector was able to transfer an exogenous gene into the endothelium of both pulmonary and bronchial circulations. This implies that the Ad vector reached the bronchial circulation; this may occur via the anastomoses between bronchial and pulmonary circulations or via direct sources other than the bronchial arteries, including the pulmonary arteries.

However, the anatomy of the bronchial arteries shows great variability in humans (8), as in sheep (9). Consistent with this, the data showed variability in gene

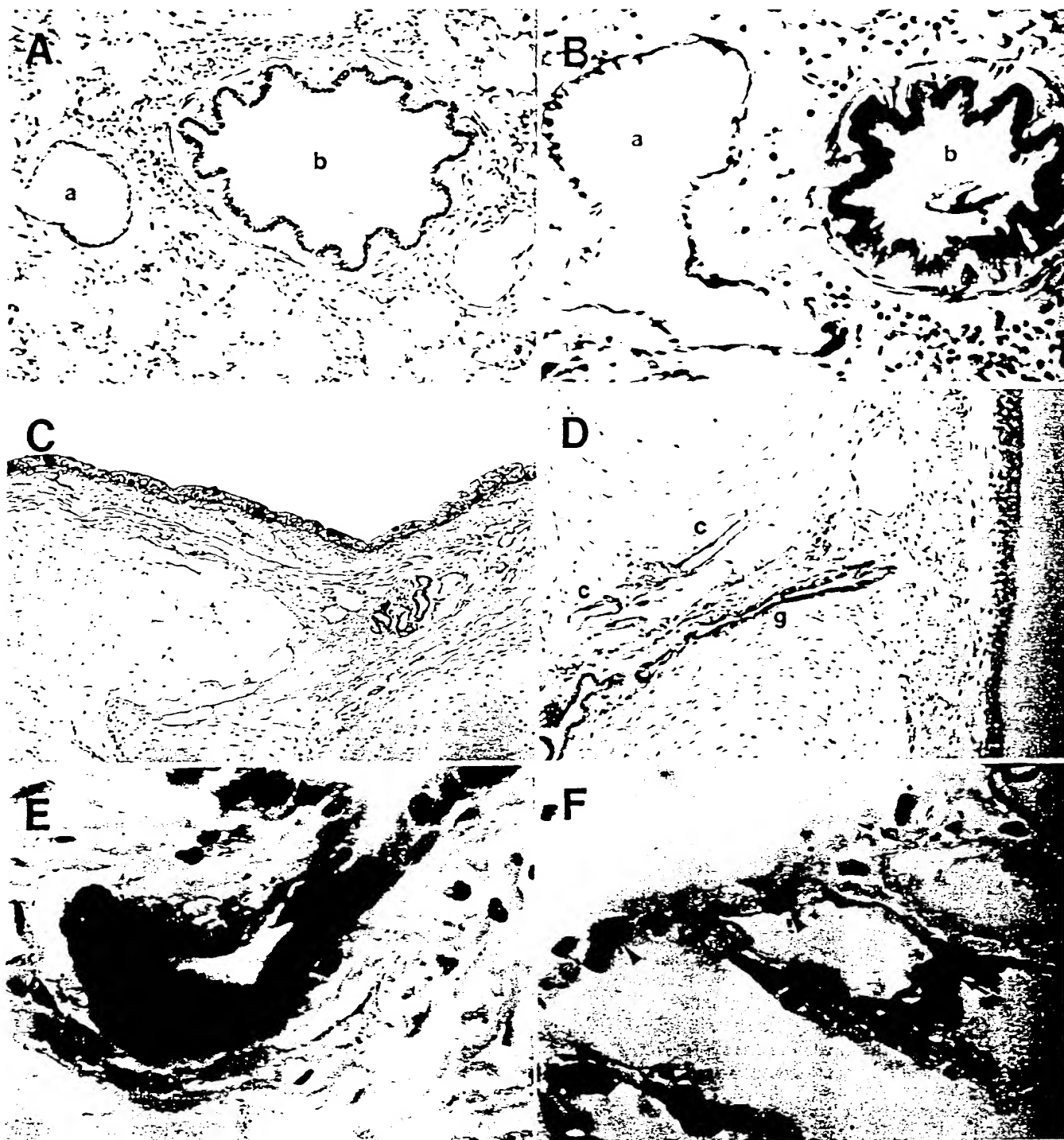


FIG. 2. Ad-mediated transfer and expression of *lacZ* gene in lung epithelium after in vivo pulmonary artery administration of vector. Ad.RSV β gal vector was administered and lungs were evaluated (as described in Fig. 1) 1 (A, B, and D-F) or 4 days later (C). Shown are photographs of paraffin sections counterstained with nuclear fast red; β -Gal activity (blue) is present in endothelium of pulmonary arteries (B; a), epithelium of bronchioles (B; b), epithelium of bronchi (C), endothelium of bronchial capillaries (D; c), epithelium of submucosal glands (D; g) and their canals (E), and epithelium of alveoli including pneumocytes type 2 (arrowhead) and type 1 (arrow) (F). A: left lung, uninfected. B-F: RUL, Ad.RSV β gal infected. A and D, $\times 200$; B, $\times 400$; C, $\times 100$; E, $\times 630$; F, $\times 1,000$.

transfer (Table 1). Because of the multiple anastomotic channels and the anatomic and functional variability, the variation in gene expression most likely reflects the variation in the anatomy of the circulation.

The way the Ad vector reaches the epithelial cells from the circulation through the endothelial junctions and the

endothelial basement membrane is unknown. The fact that Ad vectors can reach nonendothelial cells when administered through the circulation has been demonstrated in other organs. In rats, Ad vector injection to the liver via the portal vein, a site with fenestrated capillary endothelium (1), results in gene expression in hepato-

cytes but without significant expression in endothelial cells (19). Furthermore, the intravenous administration of Ad vectors in mice results in expression of the foreign gene in several cell types, including hepatocytes, myocytes, and skeletal muscle cells (15, 33). In contrast, the administration of Ad vectors in large temporarily occluded vessels such as jugular veins and carotid arteries results in localized endothelial cell gene expression (22). Similarly, in the present study β -Gal expression was limited to the endothelial layer in the pulmonary arteries. At the arteriolar and capillary levels, the Ad vector was expressed in both endothelium and adjacent airway or submucosal gland epithelium. The pulmonary endothelium, in contrast to the bronchial endothelium (20), is not fenestrated but does have numerous micropinocytotic vesicles (31) that may allow Ad to pass through the endothelium and reach the epithelial cells through their vascular surface. Because of local hypoxia during the vessel occlusion, the secretion of factors such as histamine or calcium might have also increased local extravasation of the Ad vector.

The expression of the exogenous gene in the submucosal gland epithelium after Ad vector administration in the pulmonary artery was unexpected and is of particular interest. Submucosal glands are involved in the pathogenesis of a variety of diseases, including cystic fibrosis (CF). Expression of the normal CF gene in airway lining cells is quite low, with higher expression in the submucosal glands, especially in the serous cells (12, 18, 35). Although it is not known whether the pathogenesis of CF is related to submucosal glands, it is reasonable to hypothesize that gene therapy for CF should target cells of both the bronchial epithelium and the epithelium of submucosal glands. However, if the airway delivery route is used, submucosal glands are potentially much less accessible to gene therapy than bronchial epithelial cells, as their architecture and mucus-filled lumen may provide a difficult barrier. As an alternative approach, as demonstrated in the present study, gene transfer to submucosal gland epithelium might be achieved in humans with the administration of an Ad vector into the pulmonary circulation, reaching the targeted cells through their basolateral surface. Interestingly, as in humans, the airways of the sheep contain a high number of submucosal glands that are predominantly serous (24). The feasibility of this approach as a clinical application to transfer genes to the lungs needs to be further addressed in future studies.

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RNA15 can bind to poly(U) ribopolymer (20), which suggests that the generally U-rich RNA sequences important for 3' processing in yeast (4, 21) may be recognized by this protein. The finding that this putative RNA-binding protein is a component of CF I may thus help to elucidate the sequence requirements in yeast 3'-end processing. It is worth noting in this context that the mammalian counterpart of yeast CF I, cleavage and polyadenylation specificity factor (CPSF), is a sequence-specific RNA-binding factor consisting of multiple polypeptides (1, 22).

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8. The relevant genotypes of the starting strains are as follows: LM61 (*ma14-1; pap1Δ; LEU2; ura3-1; trp1-1; ade2-1; and pAP1*) and LM62 (*ma15-1; pap1Δ; LEU2; ura3-1; trp1-1; ade2-1; and pAP1*). *pRNA14* and *pRNA15* are *TRP1*-marked low-copy plasmids containing genomic fragments allowing complementation of *ma14* and *ma15* mutations, respectively. *pApap1-5* is a low-copy *ADE2*-marked plasmid carrying the *pap1-5* mutant allele (9, 23).
9. P. J. Preker and W. Keller, unpublished results. Eight different temperature-sensitive mutant alleles, called *pap1-2* to *pap1-9*, were generated by polymerase chain reaction mutagenesis with the use of either low deoxyadenosine triphosphate (dATP) concentration or inclusion of manganese in the reaction. These mutants were sequenced, and most of them showed multiple mutations. In vitro, the extract of the *pap1-5* mutant used in this study shows approximately 50% of specific polyadenylation activity as compared with that of a wild-type extract.
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12. A standard in vitro processing reaction was done in a 25-µl reaction volume containing 2 µl of extract, 1.6 mM Hepes-KOH (pH 7.9), 0.016 mM EDTA, 4 mM potassium chloride, 1.04 mM dithiothreitol, 1.6% glycerol, 2% polyethylene glycol, 75 mM potassium acetate, 1.8 mM magnesium acetate, 2 mM ATP, 20

mM creatine phosphate, creatine kinase (0.2 mg/ml, 0.01% NP 40, and ~0.2 units of RNAGuard (Pharmacia). When only the cleavage reaction was assayed, CTP replaced ATP, and EDTA was used instead of magnesium acetate, which prevents poly(A) addition and degradation of the 3' fragment (4). The reactions were incubated at the temperatures and for the times indicated in the figure legends. They were stopped by addition of 75 µl of a stop solution [100 mM Tris-HCl (pH 8), 150 mM NaCl, 12.5 mM EDTA, 1% SDS, proteinase K (0.2 mg/ml), and glycogen (10.5 mg/ml)] and incubated for 1 hour at 42°C. The RNAs were recovered by precipitation and analyzed as described (4). For complementation assays, the extracts were mixed in a 1:1 ratio.

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15. The synthesis of the short tracts observed with *ma14* and *ma15* mutant extracts still depends on the (UA)₆ cis-acting sequences of the *GAL7* precursor, because no polyadenylation was obtained with the mutant precleaved RNA (*GAL7-7*), in which these signals were deleted (4).
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17. Fifty microliters of crude anti-serum or preimmune serum directed against RNA14p, RNA15p, or PAP1 was coupled to ~40 µl of packed protein A-Sepharose (PAS) pre-equilibrated with buffer E (4) plus 0.01% NP-40 for 3 hours at 4°C. After three washes with the same buffer, 70 µl of a wild-type extract was added to the resin and incubated for 4 hours at 4°C on a wheel. The supernatant was reappplied on a fresh antibody-PAS resin for a second round of depletion. The wild-type strain used here and for the fractionation of the 3' processing factors is a commercial brewery *S. cerevisiae* strain, referred to as VDH2 (Versuchsanstalt der Hefindustrie, Berlin, Germany). The cells were broken in a Bead Beater (BioSpec, Bartlesville, OK), and the protein extract was further prepared as previously described (4). Extracts made from this strain were active for 3' processing of *CYC1*, *GAL7*, and

18. J. Lingner and W. Keller, unpublished results. The serum was obtained from a rabbit immunized with recombinant PAP (5).
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Correction of Lethal Intestinal Defect in a Mouse Model of Cystic Fibrosis by Human *CFTR*

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Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (*CFTR*). A potential animal model of CF, the *CFTR*^{-/-} mouse, has had limited utility because most mice die from intestinal obstruction during the first month of life. Human *CFTR* (h*CFTR*) was expressed in *CFTR*^{-/-} mice under the control of the rat intestinal fatty acid-binding protein gene promoter. The mice survived and showed functional correction of ileal goblet cell and crypt cell hyperplasia and cyclic adenosine monophosphate-stimulated chloride secretion. These results support the concept that transfer of the h*CFTR* gene may be a useful strategy for correcting physiologic defects in patients with CF.

Cystic fibrosis mice bearing a null mutation in the *CFTR* gene lack adenosine 3',5'-monophosphate (cAMP)-stimulated Cl⁻ transport in intestinal epithelial cells,

which leads to goblet cell hyperplasia, intestinal obstruction, and perforation (1). To correct the lethal intestinal abnormalities in a group of CF mice, we used the rat intestinal fatty acid-binding protein (FABP) gene promoter (2) to direct expression of the wild-type h*CFTR* complementary DNA (cDNA) to the intestinal epithelial cells of these mice (3). A chimeric FABP-h*CFTR* gene construct was microinjected into fertilized oocytes, producing

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transgenic mice from both heterozygotic *CFTR*^{+/+} and wild-type FVB/N mice. The FABP-h*CFTR* transgene was detected by

Southern (DNA) blot analysis in founder mice and their offspring, and the integrity of the DNA was confirmed by restriction

fragment analysis (4). The transgenic mice were bred to produce *CFTR*^{-/-} mouse lines bearing the FABP-h*CFTR* transgene.

Fig. 1. The RT-PCR analysis of *hCFTR* mRNA. Reverse transcription was done on total tissue RNA with an oligo(dT) primer. Beta-actin cDNA was used as a control (cont). PCR of the *hCFTR* fragment was done with primers 5'-TAAACCTACCAAGTCAACCA-3' and 5'-AAT-TCCATGAGCAAATGTC-3'. Sizes of the PCR products are shown on the right. (A) Expression of *hCFTR* mRNA in the intestines of transgenic mice from six transgenic lines. Lane 1 shows a positive control: lung (Lu) cDNA from the J4 transgenic mouse bearing a lung-specific SP-C-h*CFTR* construct (10). Lane 2 shows intestinal (In) cDNA from a transgene-negative littermate. The *hCFTR* mRNA was detected in the intestines of all six transgenic lines tested (lanes 4, 6, 8, 10, 12, and 14). It was also detected in the lungs (lanes 3, 5, 11, and 13) but was not in the lungs of lines E9 or F16 (lanes 7 and 9). (B) Distribution of *hCFTR* mRNA in tissues from transgenic line A2. The *hCFTR* mRNA was detected in large amounts in the duodenum, jejunum, and ileum (lanes 9 to 11), in smaller amounts in the cecum and colon (lanes 12 and 13), and in varying amounts in the brain, lung, kidney, pancreas, and stomach (lanes 1, 2, 6, 7, and 8, respectively). Gels were stained with ethidium bromide.

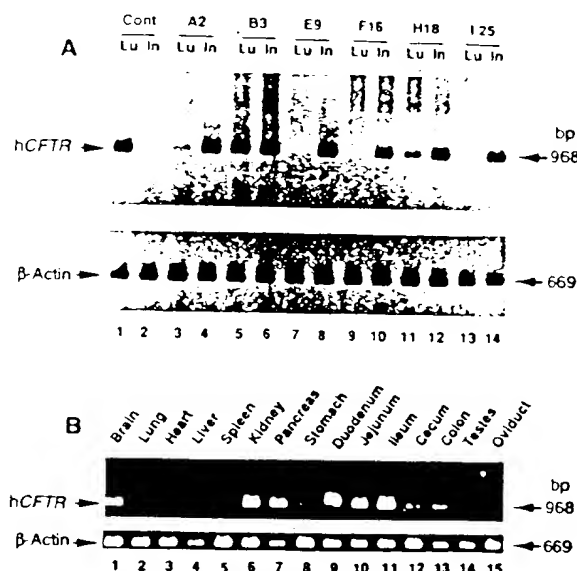
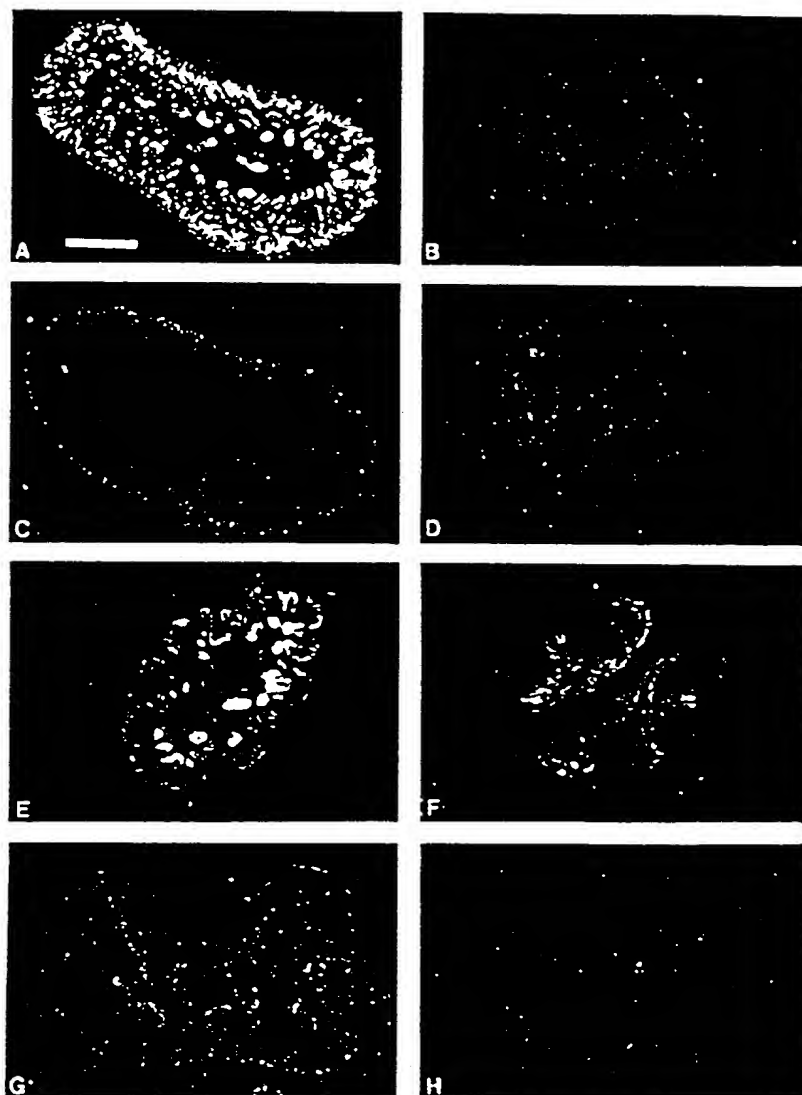


Fig. 2. In situ hybridization analysis of *hCFTR* mRNA in the adult mouse intestine. Small and large intestines from wild-type and line A2 mice were fixed in 4% paraformaldehyde. Cryostat sections (10 μ m) from the ileum (A, C, E, and G) and colon (B, D, E, and H) were hybridized overnight at 42°C with [³⁵S]UTP-labeled *hCFTR* sense and antisense riboprobes (10). The sections were then washed stringently, treated with ribonuclease A, and exposed to Ilford K5 emulsion for 7 to 10 days at 4°C. Sections were photographed under dark-field illumination. A hybridization signal was detected by antisense riboprobe in epithelial cells of the ileum (A and E) and colon (B and F) of FABP-h*CFTR*^{+/+} mice from both *CFTR*^{+/+} (A and B) and *CFTR*^{-/-} (E and F) backgrounds. No signal was detected in the ileum or colon of *CFTR*^{-/-} mice (G and H). The *hCFTR* riboprobe hybridized weakly with *mCFTR* mRNA in the crypt epithelial cells of the ileum and colon of *CFTR*^{+/+} mice (C and D). Scale bar, 500 μ m.



Human *CFTR* mRNA was readily detected by reverse transcription-polymerase chain reaction (RT-PCR) in the small intestine of six distinct FABP-h*CFTR* mouse lines (Fig. 1A). In several mouse lines, the h*CFTR* mRNA was expressed in the intestine and was absent or present in barely detectable amounts in the lung or nasal epithelium. In lines A2 and E9, h*CFTR* mRNA was most abundant in the ileum, jejunum, and duodenum and was less abundant in the cecum and colon (Fig. 1B). The h*CFTR* mRNA was not detected in the lungs of mice of the A2 or E9 lines by Northern (RNA) blot analysis but was detectable, albeit in small amounts, by RT-PCR in A2 but not E9 mice. Founder lines (A2, E9, and I25) were bred to *CFTR*^{+/-} mice, which were then bred to produce homozygous *CFTR*^{-/-} mice expressing the h*CFTR* mRNA. FABP-

h*CFTR*^{+/-}-*CFTR*^{-/-} mice from the A2 and E9 lines routinely survived weaning and showed prolonged survival (5). In contrast, 50 matings of *CFTR*^{+/-} mice from both FVB/N and C57BL/6 backgrounds resulted in survival of less than 5% of *CFTR*^{-/-} mice. Likewise, only 1 of 23 *CFTR*^{-/-} mice derived from matings of FVB/N *CFTR*^{+/-} and *CFTR*^{+/-} mice survived.

In situ hybridization demonstrated the presence of h*CFTR* mRNA in the intestinal epithelium of FABP-h*CFTR* mice from both *CFTR*^{+/-} and *CFTR*^{-/-} backgrounds (Fig. 2). The h*CFTR* mRNA was most abundant in the ileum, jejunum, and duodenum and was less abundant in the colon and cecum. It was expressed in the epithelial cells of the intestinal villi but not in the crypts of Lieberkuhn. The distribution of h*CFTR* mRNA was distinct from that of

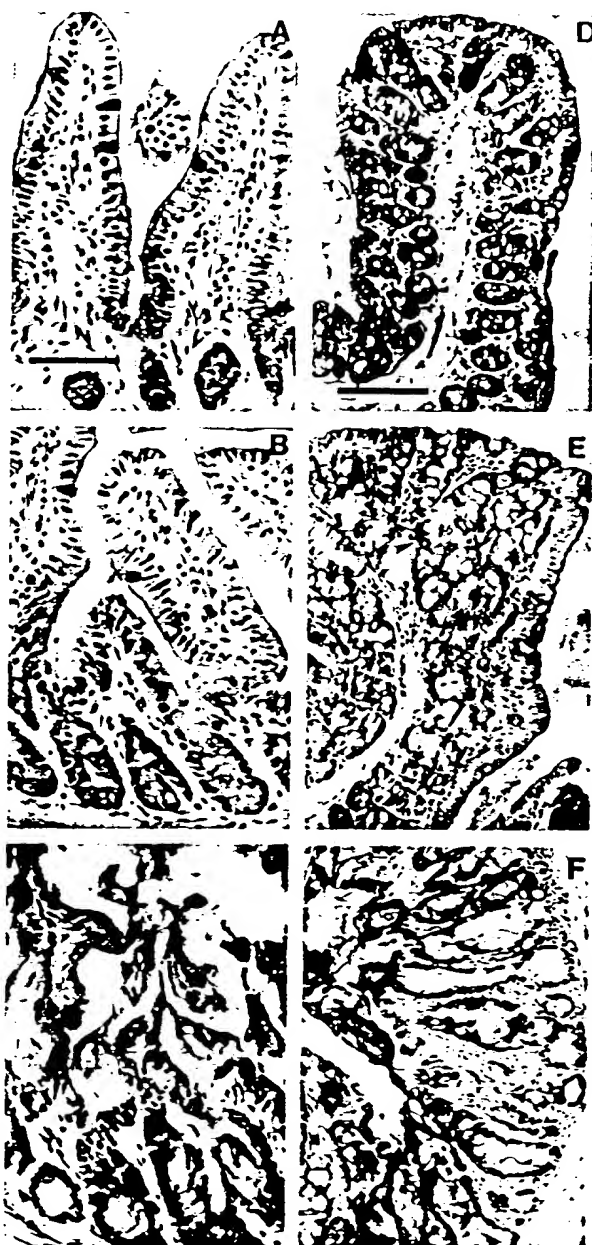
the endogenous murine *CFTR* mRNA, which was present in large amounts in the colon, ileum, and jejunum in wt mice. In these tissues, *CFTR* was expressed most prominently in the crypts of Lieberkuhn, decreased in abundance in the more mature cells along the intestinal villi, and was relatively excluded from the villous tips (6). The h*CFTR* mRNA was less abundant in the colon of the transgenic mice and, in the small intestine, was excluded from crypt cells.

Morphologic changes in the intestinal epithelium of the wild-type (nontransgenic) and *CFTR*^{-/-} and FABP-h*CFTR*^{+/-}-*CFTR*^{-/-} bitransgenic mice were further assessed by periodic acid-Schiff (PAS) staining (Fig. 3). Goblet cell hyperplasia, a prominent feature of the *CFTR*^{-/-} mice, was entirely corrected in the ileum of lines A2 and E9 FABP-h*CFTR*^{+/-}-*CFTR*^{-/-} mice. However, the disruption of crypt epithelial cell organization and goblet cell hyperplasia seen in the colon of the *CFTR*^{-/-} mice was not fully corrected in the FABP-h*CFTR*^{+/-}-*CFTR*^{-/-} mice examined (three from line A2 and one from line E9), perhaps because of inadequate expression of h*CFTR* mRNA. The coiled "wormlike" cecum that was typically observed in the *CFTR*^{-/-} mice was not observed in the FABP-h*CFTR*^{+/-}-*CFTR*^{-/-} mice examined.

Short-circuit current (*I*_{sc}) measurements were made from the intestine of *CFTR*^{-/-}, bitransgenic FABP-h*CFTR*^{+/-}-*CFTR*^{-/-}, and wt mice (Fig. 4). Forskolin-induced *I*_{sc} (rate of cAMP-stimulated Cl⁻ secretion) was absent in ileal, jejunal, and colonic segments from *CFTR*^{-/-} mice (7); phlorizin-sensitive Na⁺-dependent glucose absorption was present in the jejunum and ileum. In the small intestines of the FABP-h*CFTR*^{+/-}-*CFTR*^{-/-} mice (Fig. 4A), electrogenic Cl⁻ secretion was restored. Forskolin increased the *I*_{sc} across both jejunum and ileum of the bitransgenic animals (Fig. 4B). Addition of glucose to the mucosal solution further increased the *I*_{sc}, and this increase was phlorizin-sensitive. On average, these responses were greater in the ileum and jejunum of wt animals.

A forskolin-induced electrogenic Cl⁻ secretory response was observed in the wild-type colon but not in the colon of FABP-h*CFTR*^{+/-}-*CFTR*^{-/-} mice. This correlated with the histopathologic changes, which persisted despite upstream expression of h*CFTR* mRNA and restoration of cAMP-stimulated Cl⁻ secretory activity in the small intestine. Although the amount of h*CFTR* mRNA expression in the cecum of bitransgenics was as small as that in the colon, the cecum developed normally and did not exhibit the atrophy or irregular shape that was typical of *CFTR*^{-/-} mice. Correction of the goblet cell hyperplasia in

Fig. 3. PAS staining of ileal and colonic epithelium. Sections of the ileum (A through C) and colon (D through F) of *CFTR*^{+/-} (A and D), FABP-h*CFTR*^{+/-}-*CFTR*^{-/-} (B and E), and *CFTR*^{-/-} (C and F) mice were stained with PAS and hematoxylin. The goblet cell hyperplasia and dilation of crypts with mucus was seen in both ileal and colonic mucosa of *CFTR*^{-/-} mice (C and F) and was corrected in the ileal mucosa of FABP-h*CFTR*^{+/-}-*CFTR*^{-/-} mice (B). The goblet cell hyperplasia and distension of crypt cells (arrowhead) were still seen in some areas of the colonic mucosa of FABP-h*CFTR*^{+/-}-*CFTR*^{-/-} mice (E). Scale bar: (A through C), 64 μ m; (D through F), 128 μ m.



the ileum demonstrates the importance of CFTR expression and Cl^- secretion in the pathogenesis of the lethal obstructive phenotype in the small intestines of $\text{CFTR}^{-/-}$ mice. Our data suggest that the small amount of hCFTR mRNA in the colonic epithelium was not sufficient to fully correct the transport and histologic abnormalities in the colon of the CF mouse. In contrast, normal cecal development may depend more on luminal factors than on its CFTR-dependent ion transport functions.

The principal secretory activity of the small and large intestines resides in the undifferentiated cells of the crypts of Lieberkuhn (8), which correlates with the site of endogenous CFTR expression (6). Several features of the transport responses observed in the bitransgenic animals are consistent with expression of CFTR mRNA in the more differentiated villus absorptive cells. First, the forskolin-induced ΔI_{sc} was smaller than the wild-type response, which suggests that the spatially restricted expression of hCFTR mRNA does not quantitatively correct the Cl^- secretory response. Second, bumetanide inhibited ~60% of the forskolin-induced ΔI_{sc} in wild-type intestine but only ~30% of the ΔI_{sc} in bitransgenic animals, which suggests that the bumetanide-sensitive Na-K-2Cl cotransporter may not be the primary mechanism whereby Cl^- enters cells that express hCFTR. Third, glucose-stimulated I_{sc} was smaller in the bitransgenic animals. Glucose was add-

ed after forskolin, which would increase the apical Cl^- conductance, depolarize the apical membrane potential, and thereby reduce the driving force for Na-dependent glucose entry into villus absorptive cells. Thus, the features of the transport assays are consistent with a greater amount of hCFTR expression in villus than in crypt cells. Nevertheless, hCFTR mRNA and the Cl^- secretion rate that it supports are apparently sufficient to prevent intestinal obstruction.

Patients with CF suffer from a variety of medical complications, including severe pulmonary infections and gastrointestinal disorders that account for the increased morbidity and mortality associated with the disease (9). Meconium ileus commonly affects 10 to 20% of newborn human infants with CF and is caused by inspissated intestinal contents that cause obstruction or perforation of the bowel in utero or postnatally. It is encouraging that the lethal phenotype associated with the lack of the CFTR gene in the small intestine can be fully corrected by transfer of the hCFTR cDNA in a tissue-selective manner and that correction can be achieved even though the pattern of FABP promoter-driven expression differs from that of endogenous CFTR. These results provide further support for efforts to treat CF by gene therapy. The FABP-hCFTR $^{+/-}$ -CFTR $^{-/-}$ bitransgenic mice will be useful in determining the abundance and distribution of CFTR expression that are required to correct the physiological and histologic abnormalities in

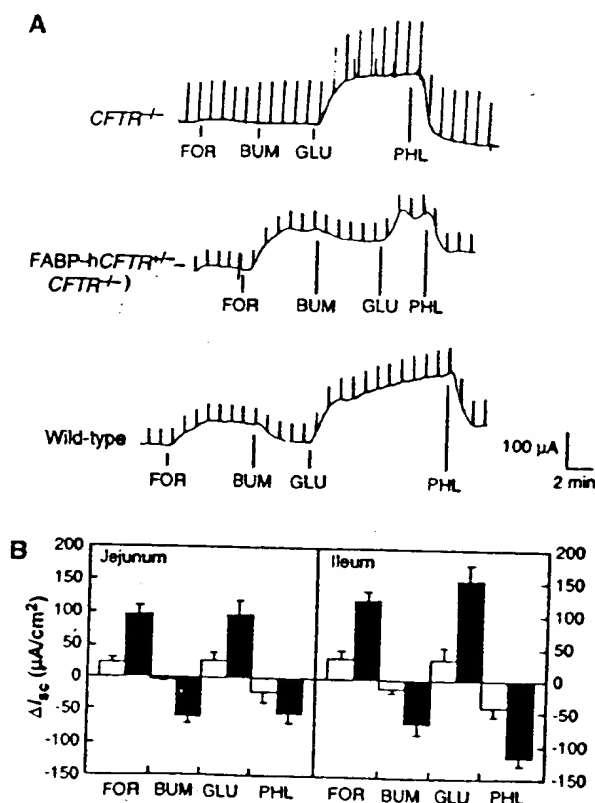
the intestine of the CF mouse and will provide a more robust model to assess the effect of the null CF mutation on the respiratory tract.

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3. A 1206-base pair (bp) portion of the 5' region of the gene encoding rat FABP, nucleotides -1178 to +28, was subcloned into pUC18, which contained a triton polyadenylate cassette. A Sal I fragment containing nucleotides 122 to 4622 of the hCFTR cDNA sequence was placed 3' to the FABP transcription element. The hCFTR cDNA fragment contained a silent T to C mutation at position 936 to stabilize the cDNA in high copy number plasmids by inactivating the cryptic bacterial promoter.
4. A chimeric FABP-hCFTR gene construct was microinjected into fertilized oocytes, producing transgenic mice from both heterozygous CFTR $^{+/-}$ and wild-type FVB/N mice. The FABP-hCFTR transgene was detected by Southern blot analysis in founder mice and their offspring, with the use of a 4.5-kb hCFTR cDNA fragment as a probe, and the integrity of the DNA was confirmed by restriction fragment analysis. The copy number varied from 4 to 84 among nine distinct founder lines produced. Wild-type and heterozygous CFTR $^{+/-}$ mice were identified by PCR (7) and were bred to establish permanent wild-type and CFTR $^{-/-}$ mouse lines bearing the FABP-hCFTR transgene.
5. Matings of mice from line A2 that were from FABP-hCFTR $^{+/-}$ -CFTR $^{-/-}$ produced 101 offspring, of which 29 were homozygous CFTR $^{-/-}$ and bore the FABP-hCFTR transgene. Eight FABP-hCFTR $^{+/-}$ -CFTR $^{-/-}$ mice were killed for study (at age 1 to 3 months) and were found to have been well. None had developed intestinal obstruction at ages ranging from 1.5 to 7.5 months. All FABP-hCFTR $^{+/-}$ -CFTR $^{-/-}$ mice from line E9 (9 of 22 total offspring) were well at ages ranging from 1 to 4.5 months. FABP-hCFTR $^{+/-}$ -CFTR $^{-/-}$ mice from I25 were not fully corrected; one died at 8.5 months from colonic obstruction, and others died at 1 to 2 months of age. Lines derived from A2, E9, and I25 bred well. Both male and female FABP-hCFTR $^{+/-}$ -CFTR $^{-/-}$ mice from lines A2 and E9 were fertile.
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11. Tissue segments 5 mm in length were mounted on plastic adaptor rings and inserted into modified Ussing chambers with an exposed area of 0.1 cm². The standard bathing solution (37°C) contained 116 mM NaCl, 1.2 mM MgCl₂, 25 mM NaHCO₃, 1.2 mM NaH₂PO₄, 10 mM mannitol (mucosal), and 10 mM glucose (serosal) (pH 7.4). The I_{sc} was monitored continuously; the transepithelial conductance was determined periodically by measuring the current needed to clamp the transepithelial potential to +1 mV (+2 mV for the CFTR $^{-/-}$ I_{sc} trace in Fig. 4A). Adjacent jejunal segments were taken from the middle portion of the small intestine. Adjacent ileal segments were taken from the distal small intestine immediately proximal to the ileocecal junction.
12. We thank X.-Y. Hu for technical assistance and B. Koller for providing CFTR $^{-/-}$ mice. Supported by the Cystic Fibrosis Foundation and by grants from NIH (HL51832 (from the Center for Gene Therapy for Cystic Fibrosis), DK38518, and HL49004).

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Fig. 4. (A) The I_{sc} recordings from ileal tissues. Sequential additions of 5 μM forskolin (FOR, both solutions), 100 μM bumetanide (BUM, serosal), 5 mM glucose (GLU, mucosal), and 200 μM phlorizin (PHL, mucosal) were as shown. (B) Mean ΔI_{sc} responses from jejunal and ileal tissues from FABP-hCFTR $^{+/-}$ -CFTR $^{-/-}$ ($n = 3$; open bars) and wild-type ($n = 5$; solid bars) mice (11). The bars represent the mean \pm SE of three to five tissue segments per mouse. The ΔI_{sc} represents the maximal response to addition of forskolin, bumetanide, glucose, and phlorizin.



Efficiency of gene transfer for restoration of normal airway epithelial function in cystic fibrosis

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An important issue for *in vivo* gene therapy for cystic fibrosis (CF) is the percentage of cells within the CF airway that will require correction. In this study, we mixed populations of a CF airway cell line expressing either the normal cystic fibrosis transmembrane conductance regulator (CFTR) cDNA (corrected cells) or a reporter gene in defined percentages. As few as 6–10% corrected cells within an epithelial sheet generated Cl⁻ transport properties similar to sheets comprised of 100% corrected cells. Cell–cell coupling may serve as the mechanism for amplification of the functional effects of corrected cells. These data suggest that *in vivo* correction of all CF airway cells may not be mandatory.

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Cystic fibrosis (CF) is the most common lethal genetic disease among caucasians¹. The disease has an autosomal recessive pattern of inheritance and causes abnormal electrolyte transport in airway, sweat ductal, intestinal and pancreatic ductal epithelia^{1,2}. The most prevalent defect associated with CF is abnormal regulation of the transepithelial chloride (Cl⁻) transport by cAMP^{3–6}. Introduction of the normal CFTR cDNA into a CF pancreatic cell line using retroviruses⁷ and into a CF airway epithelial cell line using vaccinia virus vectors⁸ has been shown to correct the Cl⁻ transport defect. Normal CFTR cDNA will restore Cl⁻ transport to CF cells functioning as a polarized epithelial sheet⁹. Moreover, phenotypic correction of the Cl⁻ transport defect can persist for up to six months in these transformed airway cells⁹. Recently, complementation of the CF Cl⁻ transport defect *in vitro* and efficient delivery of the CFTR cDNA to cotton rat lung epithelia *in vivo* has been reported using an adenovirus vector¹⁰.

All of the correction studies thus far have used efficient transfer of the normal CFTR cDNA to CF cells through either the use of selectable markers or the natural tropism of the virus for a particular cell type^{7–10}. Gene transfer using retroviruses is attractive because of the stable association of the transferred genetic material with the host genome. However, retrovirus-mediated gene transfer *in vivo* will likely be less efficient because of the requirement of proliferating cells, coupled with the inability to select for cells expressing genes of interest. Moreover, in the diseased lung, gene transfer efficiencies of 100% will be difficult to achieve with any method of gene transfer. Hence, it becomes important to determine certain minimal

requirements for gene therapy for CF to be efficacious *in vivo*. We approached this issue *in vitro* using retrovirus-mediated gene transfer into immortalized CF airway epithelial cells.

Bioelectric characterization of Cl⁻ transport

We determined the chloride transport properties of polarized airway epithelial sheets containing defined proportions of CFTR-corrected cells. The percent inhibition of the basal short circuit current (I_{sc}) by amiloride is shown in Fig. 1a. In normal or corrected airway epithelia, the Na⁺ channel blocker, amiloride, abolishes the basal Na⁺ transport and induces Cl⁻ secretion at a rate that is proportional in part to the apical membrane Cl⁻ conductance^{11,12}. In CF, amiloride abolishes Na⁺ transport, but no Cl⁻ secretion is induced because of the low or absent basal Cl⁻ conductance^{12,13}. Virtually complete inhibition of I_{sc} occurred in the interleukin 2 receptor (IL2R)-marked CF cell group (0% corrected cells) in response to amiloride, consistent with the absence of a significant basal Cl⁻ conductance. Similar findings were observed in the 1% corrected cell group. In contrast, the presence of 10% corrected cells was associated with substantial reduction of the percent amiloride inhibition of I_{sc} , reflecting the expression of a basal Cl⁻ conductance. This percentage inhibition of the I_{sc} for 10% corrected cells was not different from that seen in the 30, 50, 67 and 100% corrected cell groups.

The change in transepithelial potential difference (ΔV_t) in response to luminal Cl⁻ substitution was also consistent with a requirement for a low percentage of corrected cells to achieve phenotypic restoration of normal Cl⁻ transport

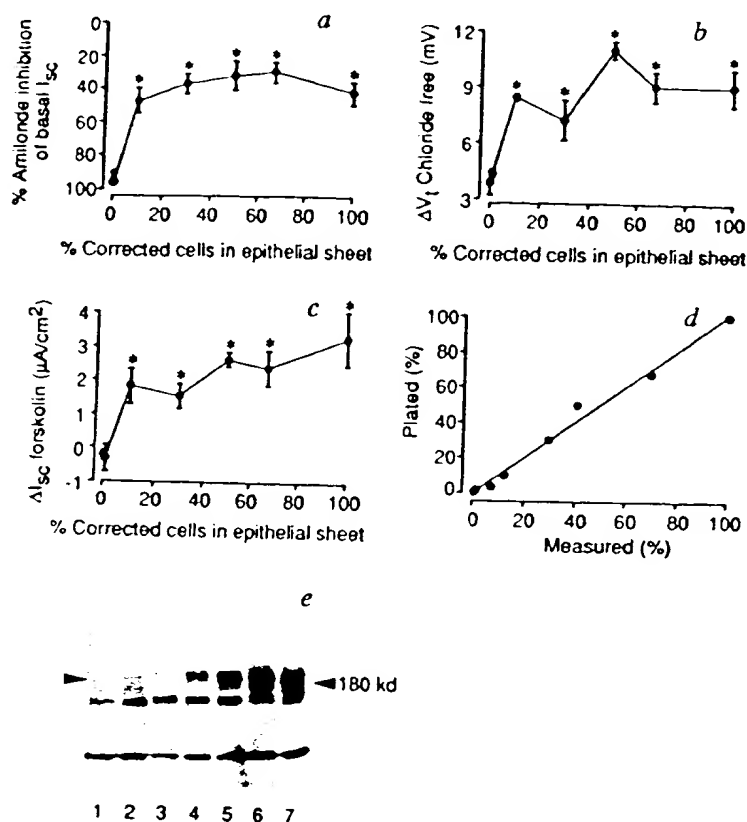


Fig. 1 Bioelectric characterization of apical membrane Cl⁻ transport in epithelial sheets. All data are presented as the mean \pm s.e. Percentage inhibition of the basal I_{sc} after 10⁻⁴ M amiloride addition to the luminal bath (basal I_{sc} - residual I_{sc} / basal I_{sc} \times 100) plotted against the percentages of corrected cells. The basal I_{sc} in units of μ A/cm² for each corrected cell group is as follows: 0%, 15.5 \pm 1.4; 1%, 13.9 \pm 3.1; 10%, 12.6 \pm 1.9; 30%, 14.6 \pm 1.6; 50%, 16.6 \pm 2.7; 67%, 17.8 \pm 3.2; and 100%, 22.0 \pm 3.7. *b*, Change in transepithelial potential difference (ΔV_t) following replacement of the luminal Cl⁻ with gluconate. *c*, Change in I_{sc} in response to 10 mM forskolin added to both the basal and luminal baths plotted against the percentage corrected cells. *d*, Quantitation of the percentages of corrected cells determined at the time of characterization of Cl⁻ secretory capacity (6 days after plating) plotted against the percentages of corrected cells plated. *e*, Western blot for CFTR immunoreactive protein. Shown is the expected mobility of CFTR (180K) for each of the corrected cell groups. Lanes are as follows: 1) 0%, 2) 1%, 3) 10%, 4) 30%, 5) 50%, 6) 67%, and 7) 100% corrected cells. Statistical significance was determined at the 95% confidence level using a Student's *t*-test with a Bonferroni correction. Asterisk indicates significantly different from 0% corrected cells (IL2R-marked CF cells).

(Fig. 1*b*). In normal airway or corrected CF airway epithelial cells, luminal Cl⁻ substitution generates a bi-ionic potential that reflects in part the relative Cl⁻ permselectivity of the apical membrane^{11,12}. In uncorrected CF airway epithelial cells, the apical membrane exhibits no Cl⁻ conductance^{12,13}, and the ΔV_t in response to Cl⁻ substitution is consequently reduced. Like the percentage inhibition of I_{sc} by amiloride, $\Delta V_{t, Cl^- free}$ in the 10% or greater percentages of corrected cells was significantly greater than $\Delta V_{t, Cl^- free}$ in the IL2R-marked CF cell (0% corrected) and 1% corrected cell groups, but was not different from ΔV_t in the 100% corrected group.

The change in short circuit current (ΔI_{sc}) induced by the cAMP-mediated agonist forskolin, was used to assess regulation of the apical membrane Cl⁻ conductance. As observed in freshly excised CF airway epithelia *in vitro* and

in heterologous cultures of CF airway epithelial cells^{14,15}, no significant change in I_{sc} occurred in response to forskolin in the 0% corrected group (Fig. 1*c*). In the 10% corrected group, the response to forskolin detected was significantly greater than that observed in the 0% corrected cell group, but not different from the 100% corrected group. The 1% group was not different from the 0% group.

Quantitation of corrected cell percentages

The validity of this series of experiments rests on quantitation of the percentages of corrected cells in the monolayer at the time of bioelectric characterization. Because our IL2R-marked CF cell and corrected cell lines originated from the same passage number of the same clonal cell line, we would expect the growth rates of these two cell lines to be similar. We used the reporter gene in our IL2R-marked CF cell line to address this point directly. By immunofluorescence staining for IL2R, the percentage IL2R-marked CF cells within epithelial sheets that were utilized for bioelectric studies could be identified and the percentage of corrected cells calculated by subtracting the percentage IL2R-marked CF cells from 100%. An excellent correlation (Fig. 1*d*) was seen between plated and actual percentages of corrected cells at the time of bioelectric studies (~6 days later). Moreover, a western blot for CFTR immunoreactive protein performed on 2% SDS lysates of these epithelial sheets demonstrated a linear correlation between the amount of CFTR protein and increasing percentages of corrected cells (Fig. 1*e*).

Consequences of low correction percentages

Because our initial experiments did not define a threshold of correction between 1 and 10% corrected cells (Fig. 1), we performed a series of experiments to determine the minimal percentage of corrected cells required to restore Cl⁻ transport (Fig. 2). A significant increase in the voltage response to luminal Cl⁻ substitution was seen with as few as 3% corrected cells reflecting the induction of a basal Cl⁻ conductance (Fig. 2*a*). Similarly, a significant increase was seen in response to the cAMP-mediated agonist forskolin in as few as 6% corrected cells (Fig. 2*b*), consistent with normal Cl⁻ conductance regulation. Western blot analysis again demonstrated a linear correlation between the percentage of corrected cells within an epithelial sheet and immunoreactive CFTR protein (data not shown).

Effects of increasing corrected cells

In a separate series of experiments, we examined the asymptotic relationship between increasing percentages of corrected cells and Cl⁻ transport. Intracellular Cl⁻ selective microelectrode techniques were used to estimate directly the apical membrane Cl⁻ conductance, focusing on 10% and 100% corrected cells. As shown above, the percent inhibition of I_{sc} by amiloride in the 10% corrected group was significantly less than observed in the IL2R-marked CF cells (0% corrected), but not different from the 100% corrected group (Fig. 3*a*). The rate of Cl⁻ loss from cells in response to reduction of Cl⁻ in the luminal bath from 120 to 3 mM was studied as an index of the apical membrane Cl⁻ permeability (Fig. 3*b*). The rate of Cl⁻ efflux across the apical membrane in the 10% corrected group (1.28 \pm 0.31 mM min⁻¹) was different from the 0% corrected group (0.04 \pm 0.24 mM min⁻¹), but not different from the 100% corrected group (1.02 \pm 0.09 mM min⁻¹). These data suggest that the apical membrane Cl⁻

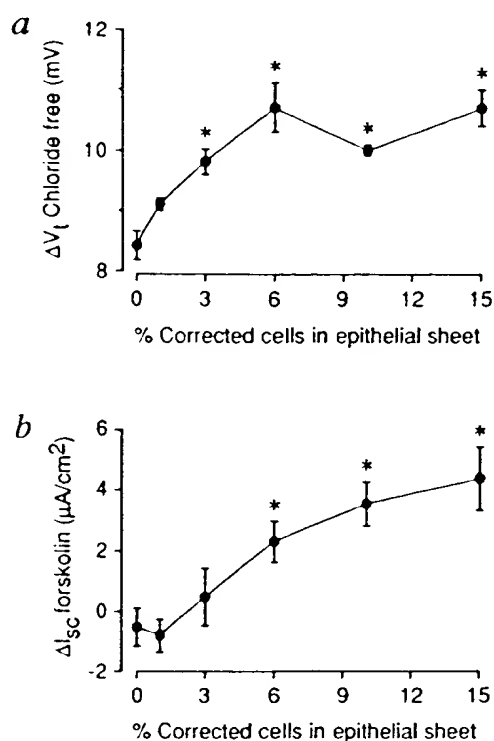


Fig. 2 Bioelectric characterization of Cl^- transport in epithelial sheets. Figure shows data from the series of experiments examining low percentages of corrected cells as follows: 0, 1, 3, 6, 10, and 15% corrected cells ($n=4-9$ for each culture group). a, ΔV_l with luminal Cl^- substitution. b, ΔI_{sc} in response to forskolin. The difference in the actual magnitude of ΔV_l between these experiments and those shown in Fig. 1 reflects the use of epithelial sheets that exhibit lower transepithelial resistances ($58.0 \pm 8.6 \Omega \cdot cm^2$ versus $155 \pm 25 \Omega \cdot cm^2$). Statistical significance for this series of experiments was determined at the 95% confidence level using a Student's *t*-test with a Bonferroni correction. Asterisk indicates significantly different from 0% corrected cells.

conductance saturates with increasing percentages of CFTR-corrected cells.

Cell-cell coupling

Because such a low percentage of cells was required for complete restoration of normal Cl^- transport, we hypothesized that cell-cell coupling, with intercellular flow of Cl^- ions between adjacent cells, might be the mechanism for amplification of the functional effects of corrected cells. In a series of four experiments, microinjection of lucifer yellow (2% solution) into single cells within monolayers of the 10% IL2R-expressing cells revealed dye spread to 23 ± 7 cells adjacent to the injected cell (Fig. 4) consistent with the presence of extensive gap junctions.

This study was designed to test whether correction of CF epithelial phenotype in the lung required gene transfer to a large or small percentage of the epithelial cells of the airway. Our data indicate that correction of a surprisingly low percentage of cells within a CF epithelial sheet was required to restore normal Cl^- transport (Figs 1-3). Our initial studies identified the range required for correction as between 1-10% (Fig. 1) and a subsequent series of studies resolved the range to ~3-6% (Fig. 2).

We tested whether the observed amplification of correction reflected communication between adjacent corrected and uncorrected cells in the epithelium. The lucifer yellow microinjection technique revealed the presence of extensive intercellular gap junctions (Fig. 4). It is unlikely that RNA transcripts move from cell to cell in the epithelial sheets because no change in the percentages of cells expressing IL2R measured immunocytochemically occurred during the time in culture. Low molecular weight proteins have also been shown to traverse intercellular junctions¹⁶, but it is unlikely that large integral membrane proteins like CFTR (~180K) move from cell to cell. Therefore, we speculate that ionic coupling via gap junctions, with Cl^- moving from uncorrected to corrected cells where it permeates the apical membrane, is the most plausible explanation for restoration of normal Cl^- transport with such a low percentage of corrected cells.

An unexpected observation was that adding more corrected cells above the 6-10% level, associated with a demonstrated increase in CFTR protein (Fig. 1e), did not increase the Cl^- transport function of the epithelium. With respect to the saturation of Cl^- transport observed during amiloride (Figs 1a, 3a), it is conceivable that the rate limiting barrier for Cl^- secretion shifts from the apical to the basolateral membrane after a finite Cl^- permeability is expressed on the apical membrane. However, the V_l response to Cl^- substitution directly measures the relative

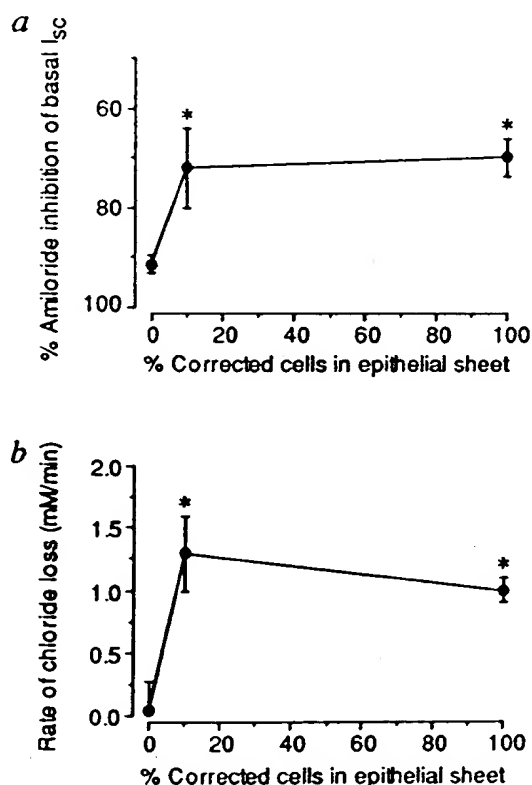


Fig. 3 Estimation of apical membrane Cl^- conductance by Cl^- selective microelectrode techniques. a, Percent inhibition of I_{sc} by amiloride plotted against the percentage of corrected cells. b, Rate of intracellular Cl^- loss against the apical membrane in response to luminal Cl^- replacement. Asterisk indicates significantly different from 0% corrected cells by students *t*-test, $p < 0.05$.

apical membrane Cl^- permeability and exhibits the same asymptotic behaviour at higher percentages of corrected cells (Fig. 1b). Furthermore, direct estimates of the apical membrane Cl^- permeability ($P_{\text{Cl}}^{\text{apical}}$) with intracellular Cl^- selective microelectrodes (Fig. 3b) demonstrated that $P_{\text{Cl}}^{\text{apical}}$ remains constant despite adding increasing numbers of corrected cells (10–100%) to the preparation. These data indicate that despite adding more individual cells expressing wild type CFTR to the epithelial preparation, the apical membrane Cl^- conductance saturates and remains rate limiting for Cl^- transport. We do not know why this saturation of apical membrane Cl^- conductance occurs. It seems likely that a well-coupled epithelium may possess homeostatic mechanisms, for example, a response to cell swelling due to increased anion permeability, to regulate (in this case downwards) the apical Cl^- conductance to physiologically relevant levels.

Extrapolation of these *in vitro* findings to gene transfer *in vivo* depends on analysis of several aspects of our system. First, the CFT1 cell appears to be a good model of airway epithelial function because it expresses ion transport rates/regulatory patterns and CFTR at levels similar to mature airway epithelia (Yankaskas, J.R. *et al.* submitted)¹⁷. In addition, the apical membrane Cl^- permeability defect observed in CFT1 cells is typical of that observed in microelectrode studies of freshly excised CF airway epithelia¹⁸ which are dominated by ciliated cells. These data suggest that ciliated cells need to be corrected. It is still not known whether secretory cells, clara cells or submucosal gland cells will also require correction. Second, the retroviral expression of CFTR achieved in this study is typical for CFTR expression from the viral LTR, in other words no selection for CFT1 clones expressing high levels of CFTR was performed. Moreover, unlike ΔF508 CFTR which does not reach the plasma membrane due to abnormal intracellular processing¹⁹, the retrovirally expressed CFTR matures normally and reaches the apical membrane where it functions normally^{9,17}. Third, evidence for cell–cell coupling, that is, gap junctions similar to that in CFT1 cells, has been demonstrated by freeze fracture in explants and epithelial outgrowths of rabbit tracheas²⁰ and in freshly excised guinea pig²¹, ferret²² and fetal lamb tracheas²³. Gap junctions have also been identified immuno-histochemically in normal human nasal epithelium using gold-conjugated antibodies to peptide fragments (α_s , β_s , β_j) and transmission electron microscopy (J.L. Carson, personal communication). Thus, it appears likely that only a low percentage of corrected cells will be required for normal Cl^- transport *in vivo*. This notion can be tested directly in CF animal models when they become available.

Methodology

Retroviral vectors/infections. Murine amphotropic retrovirus vectors were generated as described²⁴. These retroviruses contained either the CFTR cDNA or an IL2R reporter gene plus a dominant selectable *neo* gene. Immortalized CF airway epithelial cells (CFT1), derived from the tracheal epithelium of a patient homozygous for the ΔF508 deletion and transformed by the E6 and E7 genes of human papilloma virus (Yankaskas, J.R. *et al.* submitted), were infected with these amphotropic retroviruses in the presence of $4 \mu\text{g ml}^{-1}$ polybrene. CFT1 cells infected with retrovirus were selected with $150 \mu\text{g ml}^{-1}$ G418 (active) to create homogeneous populations of cells expressing either the normal CFTR cDNA (corrected cells) or the reporter gene IL2R (IL2R-marked CF cells). This IL2R-marked CF cell line, which can be identified in culture by anti-IL2R antibodies (Amersham), retains the CF Cl^- transport defect, while the corrected

cell line exhibits Cl^- transport similar to normal human airway epithelia⁹.

Bioelectric characterization of Cl^- transport in epithelial sheets. Corrected cells expressing the normal CFTR cDNA were mixed with IL2R-marked CF cells expressing the reporter gene, IL2R, in the following percentages: 0, 1, 10, 30, 50, 67, and 100% corrected cells ($n=7-9$ for each culture group from two separate experiments). These mixed populations of cells were plated on permeable collagen substrates, differentiated into polarized epithelia sheets using Swiss 3T3-conditioned tissue culture medium, and at the time of maximal transport activity, mounted in modified Ussing chambers interfaced to electrometers (Model VCC600 Voltage-current clamp; Physiologic instruments, San Diego, California) and chart recorders. Cultures once mounted were bathed on both sides with Krebs Ringer Bicarbonate (KRB) solution, pH 7.4, gassed with 95% O_2 –5% CO_2 mixture. V_i was clamped to zero and I_{sc} monitored continuously except for measurements of the spontaneous V_i across the epithelial sheet at ~5 min intervals. The transepithelial resistance of cultured airway epithelial preparations is dominated by the paracellular path such that the effect of changes in apical membrane Cl^- permeability have minimal effect on transepithelial resistance¹¹⁻¹³. The Cl^- transport responses were characterized by measuring the bioelectric responses to 10^{-4} M amiloride added to the luminal bath, luminal Cl^- replacement with 4.5 mM Cl^- containing Ringer with 100 mM K^+ substituted for Na^+ , and 10 μM forskolin.

Microelectrode studies were performed using double-barreled Cl^- selective microelectrodes interfaced to an electrometer (Model FD223 current-voltage clamp, World Precision Instruments) and a strip chart recorder as previously described¹¹⁻¹³. Epithelial sheets were mounted in modified Ussing chambers where the intracellular Cl^- activity was measured under basal conditions, in response to luminal amiloride, and in response to luminal Cl^- substitution (120 mM to 3 mM, Cl^- substituted by gluconate).

Quantitation of IL2R-marked CF cells versus corrected cell percentages within epithelial sheets. To estimate the percentages of corrected and IL2R-marked CF cells within an epithelial sheet at the time of bioelectric characterization, cells were quantitated directly by IL2R antibody staining after removal from the collagen substrate from each defined culture group. Cells were removed from culture substrates by incubation with 0.1% trypsin with 1 mM EDTA, washed and stained in suspension with a 1:100 dilution of mouse anti-IL2R antibody and a 1:400 dilution of phycoerythrin conjugated goat antimouse IgG (Biomedica Corp., Foster City, California). This cell suspension was then analysed for the percentage IL2R-expressing cells using flow cytometric analysis or by counting IL2R positive cells using a fluorescence microscope. In some cases, cells removed from the culture substrates were plated on Lab-Tek slides (Nunc Inc., Naperville, Illinois), fixed with 4% paraformaldehyde, and stained with mouse anti-IL2R followed by a goat antimouse streptavidin-biotin conjugated antibody (LSAB kit, Dako). The percentage of IL2R positive cells was quantified under light microscopy by examining five 100 \times fields for each defined culture group. The percentage of IL2R positive cells in each group was normalized relative to the percentage of positive cells measured in the IL2R-marked CF cell group which consisted of 100% cells from the CFT1 cell line expressing IL2R. The percentage of corrected cells within an epithelial sheet is defined as the total cell population within the epithelial sheet (100%) minus the percentage IL2R-marked CF cells.

Western blots. Western blots were performed as described¹⁷. Samples were harvested directly from the permeable collagen substrate by the application of 40 μl 2% SDS directly to the cell layer. The solution was then removed from the substrate, boiled for 5 min and stored at -20°C . Samples were then mixed with disaggregation buffer (50 mM Tris, pH 6.8, with H_2PO_4 , containing 2% (w/v) SDS, 15% (w/v) glycerol, 2% (v/v) β -mercaptoethanol, 1 mM EDTA, and 0.02% (w/v) bromophenol blue). Equal amounts of protein (50 μg per well) were loaded onto a 4–15% polyacrylamide gel and electrophoresis was performed at 110V for 60 min. Blotting onto PVDF membranes (Bio-Rad, 0.2 micron pore size) and immunodetection with anti-CFTR C-terminus antibody was performed¹⁷. Amersham Rainbow prestained standards and Bio-Rad high molecular mass standards were used to estimate molecular weights.

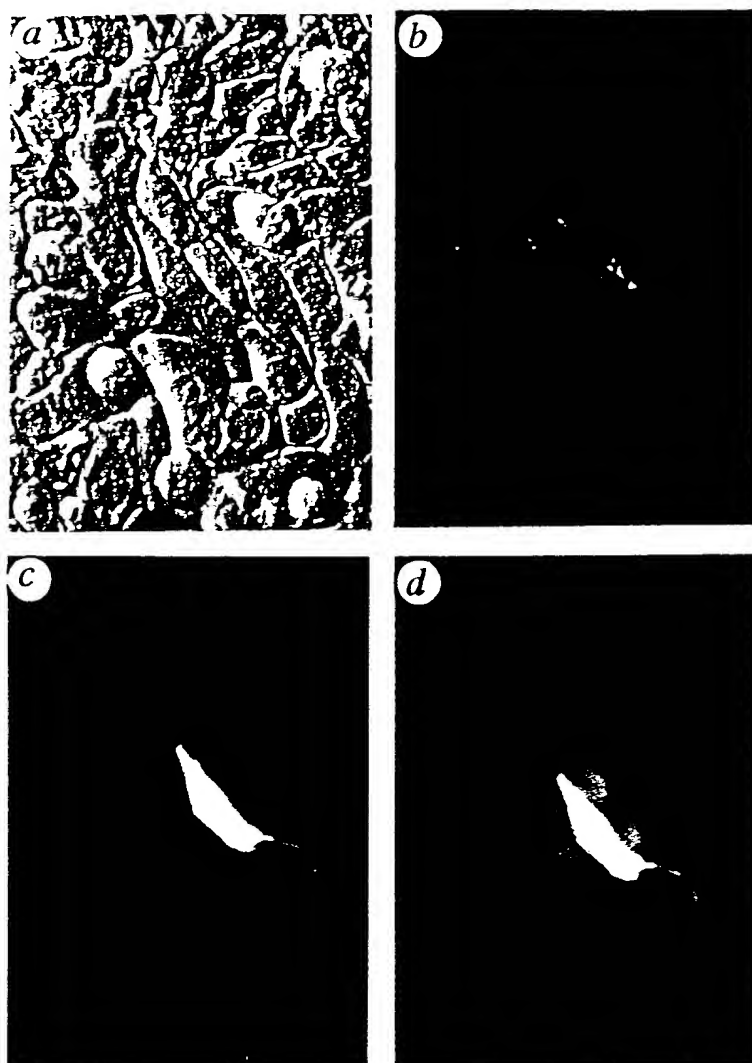


Fig. 4 Assessment of cell-cell coupling in epithelial sheets. a, Nomarsky image of CFT1 cells expressing IL2R and CFT1 cells expressing CFTR. The cell which was subsequently microinjected is marked with an asterisk. b, Same field under fluorescence microscopy showing two cells stained for IL2R. c, IL2R-positive cell immediately after microinjection with lucifer yellow. d, The same field 20 min after microinjection of lucifer yellow into the single IL2R positive cell.

Assessment of cell-cell coupling in epithelial sheets. Cultures composed of 10% IL2R-expressing cells were plated on a collagen coated permeable nitrocellulose substrate and on plastic and grown under differentiating conditions (see text). The cultures were stained for IL2R positive cells with anti-IL2R and fluorescent antibodies. Using fluorescence microscopy, a 2% solution of lucifer yellow was injected into a single IL2R positive cell using a micropipette (1.0 mm Quik-Fil, World Precision Instruments, Sarasota, Florida) interfaced to a pressure injector (Eppendorf Model 51). Photographs of the injected cells were taken immediately after injection and 20 min later. The number of cells containing dye was quantitated visually.

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Recovery of Airway Cystic Fibrosis Transmembrane Conductance Regulator Function in Mice with Cystic Fibrosis After Single-Dose Lentivirus-Mediated Gene Transfer

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ABSTRACT

The potential for gene therapy to be an effective treatment for cystic fibrosis (CF) airway disease has been limited by inefficient gene transfer vector particle delivery and lack of persistent gene expression. We have developed an airway conditioning process that, when combined with a human immunodeficiency virus (HIV)-derived lentivirus (LV) vector, resulted in persistent *in vivo* expression of transgenes in airway epithelium. Pretreatment of mouse nasal epithelium with the detergent lysophosphatidylcholine (LPC) prior to instillation of a single dose of an LV vector carrying the *LacZ* marker gene produced significant *LacZ* gene expression in nasal airway epithelium for at least 92 days. Transduction of the cystic fibrosis transmembrane conductance regulator (CFTR) gene using the same LV vector system resulted in partial recovery of electrophysiologic function in the nasal airway epithelium of CF mice (*cfr*^{tm1Unc} knockout) for at least 110 days. This first demonstration of LV-mediated *in vivo* recovery of CFTR function in CF airway epithelium illustrates the potential of combining a preconditioning of the airway surface with a simple and brief LV vector exposure to produce therapeutic gene expression in airway.

OVERVIEW SUMMARY

When used in an airway pretreatment conditioning regimen, two detergents (polidocanol and lysophosphatidylcholine [LPC]) facilitated lentivirus (LV)-mediated gene transfer to mouse nasal airway epithelium. LPC was the more effective conditioning agent, and when delivered 1 hr before the instillation of a VSV-G pseudotyped *LacZ* lentiviral vector, its use resulted in significant and long-term *LacZ* gene expression. Exclusion of polybrene from LV vector preparations improved the level of gene transfer *in vivo*. In cystic fibrosis (CF) mice, airway pretreatment with LPC followed by delivery of a cystic fibrosis transmembrane conductance regulator (CFTR) LV vector produced extended functional gene transfer; specifically, significant functional CFTR recovery remained after 46 days, with the mean Δ PD value reaching 54% of heterozygote Δ PD values. At later time points (110 days and 13 months posttreatment) Δ PD correction waned in the remaining two LV-treated CF mice. These findings suggest that LV vectors delivered after brief airway conditioning could provide a basis for long-term CFTR gene transfer *in vivo*.

INTRODUCTION

CYSTIC FIBROSIS (CF) is the most common life-threatening autosomal recessive disease in Caucasian populations, especially those of northern European origin (Hodson and Geddes, 1995). Although many organs are affected by the ion imbalances induced by malfunction of the cystic fibrosis transmembrane conductance regulator (CFTR), it is the chronic and progressive infective lung disease that produces the high levels of mortality and morbidity experienced by children and adults with CF. Recent studies indicate that the primary effect in the CF airway of the derangement of epithelial cell ion balance is a reduced airway surface liquid (ASL) volume (Tarran *et al.*, 2001) that ultimately results in defective mucociliary clearance, allowing inhaled bacteria to deposit, proliferate, and initiate a chronic infective/inflammatory lung disease (Knowles and Boucher, 2002).

Gene therapy for CF lung disease is based on the premise that if adequate CFTR function can be restored in the defective CF airway epithelial cells, then airway epithelial biology and overall lung function would be normalized. Airway infection should then be prevented and the morbidity and mortality as-

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sociated with CF lung disease averted. If airway progenitor cells could also be permanently transduced, then therapeutic CFTR gene expression would be sustained. Of the viral gene transfer vectors currently being developed, lentiviral (LV) vectors have particular advantages in that they can transduce both quiescent and dividing cells, provide stable and sustained gene expression, and do not appear to induce a significant host immune response (Amado and Chen, 1999).

While the conceptual basis of gene therapy for CF lung disease using gene transfer is both elegant and simple, and is easily demonstrated *in vitro* (Johnson *et al.*, 1992), practical barriers to its application *in vivo* have become apparent (Davies *et al.*, 2001; Koehler *et al.*, 2001). The highly effective airway defenses that have evolved to protect the mammalian airway epithelium against allergens, irritants, dust, viruses, and microbial pathogens (Bevins, 1999) also apply to gene transfer vectors. Airway mucus continually captures inhaled or introduced particles for removal by mucociliary clearance activity. At the apical cell surface the glycocalyx (Pickles *et al.*, 2000) can bind most vector types, further hindering vector particle entry. Finally, the epithelial tight-junctions (TJ) present another physical barrier to the delivery of gene transfer vectors to their receptors, which are located predominantly on the basolateral cell surfaces below the TJ (Bergelson *et al.*, 1997). Modulating the effectiveness of these barriers, for example by opening airway epithelial TJ to facilitate access of gene transfer vector particles to receptors located on the basolateral cell surface, is an approach to improving *in vivo* gene transfer that has only recently received attention (Parsons *et al.*, 1998; Coyne *et al.*, 2000; Johnson *et al.*, 2000; Wang *et al.*, 1999, 2000; Chu *et al.*, 2001). Our focus has been to use surface-active agents such as the synthetic detergent polidocanol (Parsons *et al.*, 1998) and the biologic detergent lysophosphatidylcholine (LPC) (Parsons *et al.*, 1999), to precondition the airway surface to make it temporarily permissive for viral gene transfer *in vivo*.

In this study we show that LPC, used as pretreatment reagent to condition the airway epithelium surface, in combination with a VSV-G pseudotyped human immunodeficiency (HIV)-1-based LV vector, permits sustained gene transfer into mouse nasal airway epithelial cells *in vivo*. Furthermore, a single LV vector dose was sufficient to produce expression of both a marker gene, and a therapeutic gene, that outlasted the generally accepted turnover time (approximately 3 months) of the airway epithelium (Borthwick *et al.*, 2001), suggesting that transduction of airway progenitor cells had occurred.

MATERIALS AND METHODS

DNA construction and virus production

The LV vector system used in this study (Fuller and Anson, 2001; D.S. Anson and M. Fuller, unpublished data) has been derived from HIV-1 and has been disassembled to prevent viral replication. The LV vector was produced by transient transfection of 293T cells (100 mm dish) with five different plasmids, i.e. 14 μ g of the LV vector plasmid, 3 μ g of pcDNA3gagpolml, 14 μ g of pHCMV-G (Yee *et al.*, 1994), 4 μ g of pCMV-*rev* (Lewis *et al.*, 1990), and 0.2 μ g of pcDNA3TAT101ml, using calcium phosphate coprecipitation (Fuller and Anson, 2001). Three different LV vector constructs were used: (1)

pB1HIVext5cpptSV40LacZppt⁺RRELTR (LVlacZa), (2) pBCKSHIVext4crrectcpptSV40LacZppt⁺ Δ LTR (LVlacZb), and (3) pBCKSHIVext4m2crrectcpptSV40CFTRppt⁺ Δ LTR (LVCFTR) (Fig. 1). The LVlacZa and LVlacZb vectors contain essentially the same HIV-1 sequence elements but differ slightly in the arrangement of these sequences. The most notable difference is the shorter length of *gag* gene sequence in the latter vector. The LVCFTR vector construct is essentially the same as the LVlacZb vector but with the CFTR cDNA sequence replacing the *LacZ* marker gene sequence. In all three vectors the transgene is under the transcriptional control of the simian virus 40 (SV40) early promoter. The LV vector supernatant collected was initially concentrated approximately 10-fold by ultrafiltration in a 50-ml stirred cell apparatus using a DIAFLO[®] Ultrafiltration membrane (500K weight cutoff, ZM500; Amicon, Inc., Beverly, MA) at 4°C and further concentrated (approximately 100-fold) by ultracentrifugation at 30,000 \times rpm for 1 hr and 35 min at 4°C in a Beckmann SW-60 rotor. The resulting LV vector pellet was typically resuspended in 200–300 μ l of phosphate-buffered saline (PBS; 1/1000th of the starting volume of the LV vector supernatant) and stored at –70°C.

Estimation of the titer of the LV vector

LVlacZ vector. NIH3T3 cells grown on 6-well tissue culture clusters were transduced with either the LVlacZa vector, or the LVlacZb vector in the presence of polybrene (4 μ g/ml). Seven days later the transduced cells were stained with 1 ml of X-gal solution (Parsons *et al.*, 1998) for 16 hr at 37°C to detect *LacZ* gene expression. The X-gal solution was then aspirated and the cell monolayer washed once with PBS. For each well the number of *LacZ*-positive cells in three 0.25-cm² squares was counted. The titer (transducing units [TU]) of the LVlacZa and the LVlacZb vectors was calculated using the following formula:

$$\begin{aligned} &\text{Number of LacZ positive cells} \times \text{dilution factor} \\ &\div \text{total surface area (9.4 cm}^2\text{)}/\text{selected surface area (0.75 cm}^2\text{)} \\ &= \text{NIH3T3-TU/ml} \end{aligned}$$

LVCFTR vector. DNA was prepared from nontransduced NIH3T3 cells (control) and NIH3T3 cells transduced with either the LVCFTR vector, or a vector of known titer, using the DNeasyTM tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The primers Ext 4F (5' GGGTGGCAGAGCGTCAGTATTAG 3') and Ext 4R (5' CTTCTCTAAAGCTTCCTTGGTGTC 3') (GIBCO, Melbourne, Australia) were designed to amplify a 306 base portion (HIV-1 YU-2, GenBank accession number M93258, bases 803–1109) of the *gag* gene sequence in the vector. Dilutions of sample and positive control (known amounts of plasmid DNA and/or DNA prepared from cells transduced with a vector of known titer) were amplified in the presence of 1 \times Taq buffer (Qiagen), 2.5 mM MgCl₂, 2 \times Q buffer (Qiagen), 200 μ M dNTPs (Roche, Adelaide, Australia), 1 μ g of Ext4 F and Ext4 R primers and 5 units of Taq (Qiagen). The reaction was heated initially to 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 60 sec, and extension at 72°C for 30 sec. The last cycle was a further extension at 72°C for 3 min. PCR products were then analyzed by

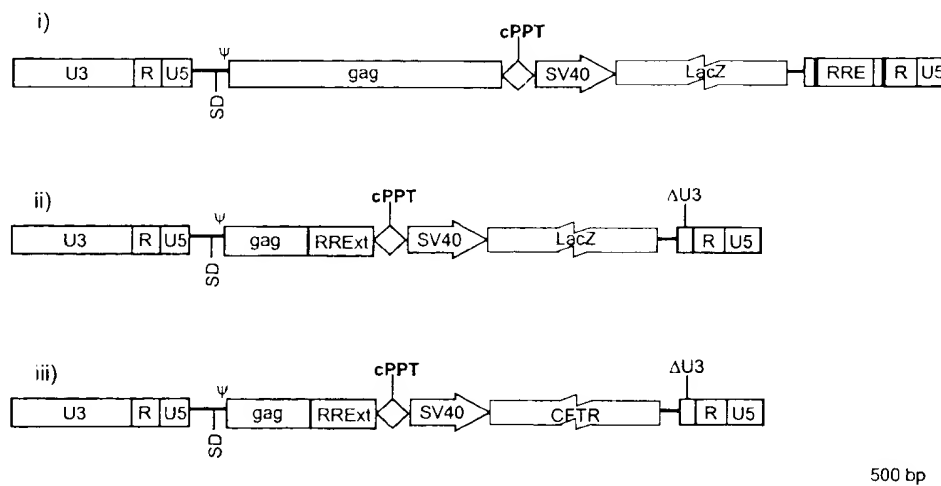


FIG. 1. Lentivirus (LV) vector constructs. **i:** The LVLacZa vector contains from 5' to 3' the human immunodeficiency virus type 1 (HIV-1) YU-2 5' viral long terminal repeat (LTR), and contiguous sequence extending 1150 base pairs into the *gag* gene, the cPPT sequence, the SV40 immediate early promoter, the *LacZ* gene sequence, the HIV-1 YU-2 polypurine tract, and the 25 base pair sequence immediately 5' of the PPT, and the 3' LTR with the rev response element (RRE) replacing sequences between the *EcoRV* and *PvuII* sites in the U3 region. This strategy renders the vector self-inactivating because vital transcriptional elements in U3 region have been replaced by the RRE sequence. **ii:** In the LVLacZb vector the length of the *gag* sequence is reduced to 550 base pairs and an extended RRE sequence is positioned immediately 5' of the cPPT rather than in the 3' LTR. The construct was made self-inactivating by deleting the sequences between the *EcoRV* and *PvuII* sites in the 3' LTR (Δ U3). **iii:** The LVCfTR vector construct is similar to the LVLacZb vector construct described above with the difference that the *gag* reading frame was blocked by mutagenesis of the ATG codons at base 788 and 1298 (HIV-1 YU-2, GenBank accession number M93258) of the HIV-1 YU-2 sequence to TAG stop codons and the CFTR cDNA sequence replaces the *LacZ* marker gene sequence.

agarose gel electrophoresis and titer calculated by comparing the amount of product from the dilutions of the experimental sample with that from the dilution series of the positive control.

Assay for detection of helper virus

In order to monitor for replication-competent retrovirus, the p24 levels present in the medium of transduced A549 cells were monitored. Briefly, A549 cells were transduced with a sample of the concentrated LV vector (LVLacZa, LVLacZb, or LVCfTR) and maintained in culture for 4 weeks. Twice a week a 1-ml sample from a confluent culture was collected and stored at -70°C until all samples were collected. The medium of non-transduced A549 cells was also collected and used as a negative control. The samples were assayed for p24 using the HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit (NEN Life Sciences, Boston, MA) according to the manufacturer's instructions. In all cases p24 declined to undetectable levels (<10 pg/ml) within 7 days of transduction.

Nasal Dosing

C57Bl/6 and *cfr^{tm1Unc}* mice (Snouwaert *et al.*, 1992) were used under the approval of both the Women's and Children's Hospital and the University of Adelaide Animal Ethics Committees. C57Bl/6 mice (6–7 weeks of age), or *cfr^{tm1Unc}* mice (8–20 weeks of age), were anesthetized intramuscularly with 1 $\mu\text{l/g}$ and 0.7 $\mu\text{l/g}$ body weight, respectively, of a 3:2 mixture of xylazil (20 mg/ml):ketamine (100 mg/ml). Body temperature was maintained during anesthesia with a heat pad or heat lamp

and during the recovery period the mice were placed in a 35°C air chamber. For dosing, mice were suspended from their dorsal incisors (hindquarters supported) and pretreatment solutions (4 μl of LPC [Sigma L-4129; Sigma, St. Louis, MO], or polidocanol [Sigma P-9641; Sigma], prepared as w/v solutions in PBS) delivered as a bolus into the right nostril using a gel-loading tip (Finnpipette, A. Daigger & Company, Vernon Hills, IL). Typically, 30 min after the initial anesthetic dose, mice were reanesthetized with half the starting anesthetic dose. One hour after the detergent pretreatment, the LV vector (or the appropriate control solution) was instilled. Two 10- μl aliquots were instilled in the right nostril over 2–3 min. The mice were monitored for respiratory distress and any loss of treatment solution was noted. Mice were weighed daily for 10 days, and observed for signs of distress over the duration of the experiment. Deaths of LV-treated CF mice in this series (Fig. 6a) were because of complications during postanesthesia recovery.

Assessment of gene transfer

LacZ gene expression. The heads were processed to reveal *LacZ* gene expression using X-gal processing as previously described (Parsons *et al.*, 1998). The types of *LacZ*-positive cells in respiratory and transitional epithelium were determined in hematoxylin and eosin (H&E)-stained cross sections, while the number of *LacZ* transduced cells was counted in three standard cross sections (Parsons *et al.*, 1998) stained with safranin O (Saf-O).

CFTR gene expression. Mice were anesthetized, suspended from their dorsal incisors (hindquarters supported), and a sub-

cutaneous needle-agar bridge (as reference electrode) was placed in the abdomen. A heat-drawn PE10 polyethylene cannula (marked with a fine-tip permanent marker at 2.5 mm and 5.0 mm to allow accurate placement of the cannula tip) was inserted to the designated depth in the treated nostril and connected to a perfusion-recording apparatus [modified dual-syringe pump (IVAC 770; IVAC Corporation, San Diego, CA), a WPI Isomillivoltmeter, and a chart recorder]. The syringe pump was loaded with two 1 ml Hamilton syringes (Hamilton Instruments, Reno, NV) containing either basal or low-chloride solution and connected to the tubing system. The cannula was inserted into the treated right nostril of the mouse at approximately 3 mm (this depth was 2 mm shallower than the 5 mm used in previous studies [Parsons *et al.*, 1998]) in an effort to improve the recording of electrical potential from only respiratory epithelium in the nose (Parsons *et al.*, 2000a). Infusion of the basal solution (approximately 2.3 μ l/min) was initiated and readings were taken until a stable TPD value was recorded (a plateau of at least 1 min was required). The infusion solution was then switched to low-chloride solution (NaCl replaced with Na gluconate) and a new TPD value recorded. Two untreated *cfr^{tm1Unc}* mice were also used for blinding purposes and as negative controls at each TPD assessment point (7, 46, 110 days, and 13 months; data not shown). The treatment category of the *cfr^{tm1Unc}* mice was blinded by tail color recoding prior to TPD recordings. TPD values were measured (blinded) from chart paper recordings, and the Δ PD value was calculated by subtracting the TPD value recorded under basal conditions from the TPD value measured under low-chloride conditions.

Statistical analysis

Statistical analysis of data was performed using SigmaStat 2.03 (SPSS, Chicago, IL). Statistical significance was set at $p = 0.05$ and a statistical power greater than 0.80 was required (if power did not reach 0.80 it is noted). Data are presented as a mean \pm standard error (SEM). Student's *t* test was used for two-group comparisons, and multiple treatment groups were analyzed by one-way analysis of variance (ANOVA) using post-test multiple comparisons to identify specific group differences. Where data did not satisfy normality assumptions, standard transformations or appropriate nonparametric methods were utilized. Changes in the proportions of transduced cell types were analyzed by logistic regression analysis using GenStat (Release 4.2, 5th edition, VSN International Oxford, UK).

RESULTS

LV-mediated LacZ gene transfer into the nasal airway epithelium

To determine the most effective detergent pretreatment regimen we first compared the effect of conditioning treatment with two doses of either polidocanol or LPC on the level of *in vivo* LacZ marker gene transduction. Groups of mice ($n = 3$) were exposed to either polidocanol (1% or 0.1%), or LPC (1% or 0.1%), 1 hr prior to instillation of 20 μ l of either the LVLacZa vector (Fig. 1) containing 6×10^4 NIH3T3-TU, or the carrier solution (PBS). To assess the effect of Polybrene on LV-mediated gene transfer, an additional group of mice were pretreated

with 1% LPC and exposed to the same dose of LVLacZa vector containing 4 μ g/ml Polybrene. Seven days later, LacZ gene expression in the nasal epithelium was revealed using the X-gal processing method.

Qualitative stereomicroscope *en face* examination of the grossly sectioned blocks of the head (prior to paraffin embedding and histologic processing) showed that 1% LPC facilitated significant gene transfer compared to PBS pretreatment. The distribution of the LacZ-positive cells, identified as scattered punctate blue-stained cells, remained ipsilateral, whereas a diffuse light green artefactual staining was distributed bilaterally. No LacZ-positive cells were seen in the control (PBS-pretreated) mice. In addition, no LacZ-positive cells were observed in olfactory regions.

When the number of LacZ-positive cells was quantified in nasal cross sections several conclusions could be made regarding our LV-mediated gene transfer protocols. First, 1% LPC pretreatment produced a fourfold increase in the level of LV-mediated transduction compared to pretreatment with 1% polidocanol, and overall the 1% LPC pretreatment provided significantly greater gene transfer than other pretreatments (ANOVA, $p < 0.05$; Table 1) including PBS (control) pretreatment (Fig. 2a). Second, inclusion of Polybrene in the LVLacZa vector preparation resulted in a fourfold decrease in the number of LacZ-positive cells (5.0 ± 1.0 vs. 19.7 ± 1.7 , $p = 0.002$). These results indicated that LPC was a more effective pretreatment reagent than polidocanol, and that the addition of Polybrene reduced the level of LV-mediated gene transduction in this *in vivo* setting. Therefore, airway conditioning with 1% LPC, and Polybrene-free LV vector preparations, were used in all subsequent *in vivo* gene transfer studies.

TABLE 1. TOTAL NUMBER OF LacZ-POSITIVE CELLS

Pretreatment	Treatment	LacZ-positive cells \pm SEM
PBS	LVLacZa	0.0 ± 0.0
1% PDOC	PBS	0.0 ± 0.0
1% LPC	PBS	0.0 ± 0.0
0.1% PDOC	LVLacZa	2.7 ± 0.3
0.1% LPC	LVLacZa	4.3 ± 0.7
1% PDOC	LVLacZa	5.3 ± 1.3
1% LPC	LVLacZa	19.7 ± 1.7
1% LPC	LVLacZa/PB	5.0 ± 1.0

C57B1/6 mice ($n = 3$ per group) were pretreated and dosed with 20 μ l of LVLacZa containing 6×10^4 NIH3T3-TU as described in Materials and Methods. Results are presented as the number of LacZ-positive cells counted in three standard cross sections. The combination of 1% LPC pretreatment and LVLacZa produced the greatest number of LacZ-positive cells in nasal airway *in vivo* ($p < 0.05$, ANOVA). In addition to the above results, no LacZ-positive cells were found in two control groups of mice ($n = 3$ each) tested by pretreatment with 4 μ l LPC pretreatment followed by 20 μ l of LVEYFP (enhanced yellow fluorescent protein, an irrelevant reporter gene in this context); and a LacZ pseudotransduction control (pcDNALacZ virus) in which a plasmid expressing LacZ was substituted for the LVLacZa.

LPC, lysophosphatidylcholine; ANOVA, analysis of variance; PB, Polybrene.

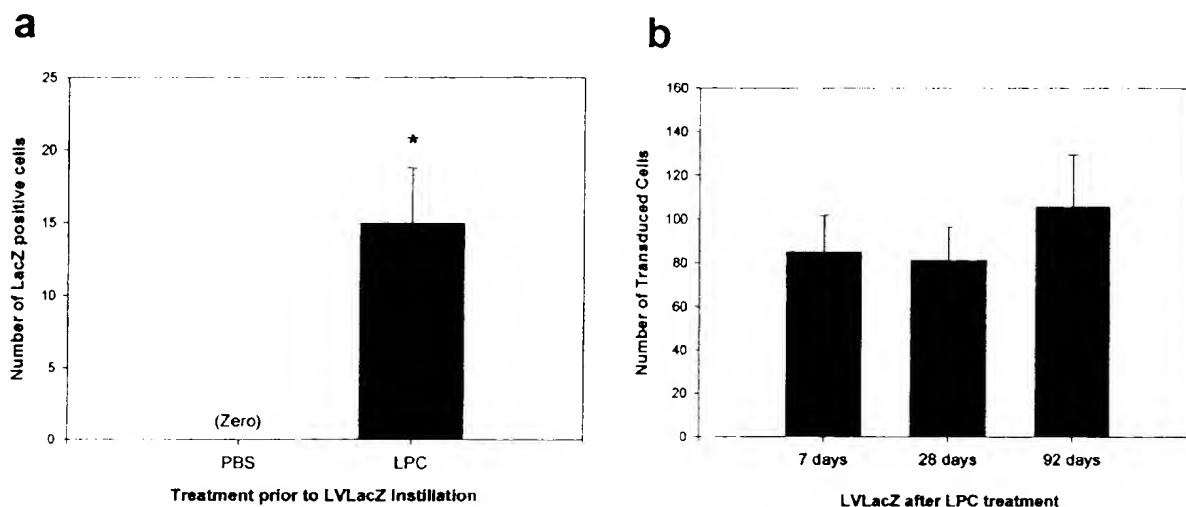


FIG. 2. Airway *LacZ* gene expression in nasal airway. **a:** Only when the LVLacZ vector instillation was combined with lysophosphatidylcholine (LPC) pretreatment (or PDOC pretreatment, not shown) was *LacZ* gene transduction observed. The combination of 1% LPC pretreatment and Polybrene-free LVLacZa vector preparation resulted in the greatest gene transfer, shown here 7 days after dosing (* $p < 0.05$, analysis of variance [ANOVA], $n = 3$ per group). **b:** Quantitation of *LacZ* gene expression over three standard nasal airway cross sections supports the qualitative impression (Fig. 3) of persistence of *LacZ* gene expression for at least 92 days after dosing. The apparent increase in transduction at 92 days was not significant ($p = 0.64$), although statistical power was low (power = 0.05, ANOVA, $n = 3$ per group).

Persistence of LacZ gene expression in the nasal airway epithelium

The level of persistence of gene expression will be a critical determinant of the utility of a vector in producing effective therapeutic CFTR gene transfer in CF airway epithelium. Therefore, we assessed the persistence of gene expression resulting from LV-mediated transduction in nasal airway epithelium. The right nostril of 9 mice was exposed to 1% LPC 1 hr prior to the instillation of 20 μ l of LVLacZb (Fig. 1), containing 1.4×10^5 NIH3T3-TU. Three treated mice were subsequently sacrificed at 7, 28, and 92 days and the heads processed for *LacZ* marker gene expression.

Qualitative *en face* examination of the blocks of tissue of the nasal airway epithelium at each posttreatment time point revealed high numbers of *LacZ*-positive cells. The distribution of these *LacZ*-positive cells at each time point remained ipsilateral along the treated nostril (Fig. 3A, 3C, and 3E) and there were also high numbers of *LacZ*-positive cells located as far posterior as the nasopharyngeal meatus (Fig. 3B, 3D, and 3F). Interestingly, although the two nasal airways (one dosed, one undosed) have coalesced into the single nasopharyngeal airway at this posterior level of the nose, *LacZ*-positive cells remained localized to the dosed side of the head. Again, no *LacZ*-positive cells were seen in the olfactory regions.

When the number of *LacZ*-positive cells was quantified in Saf-O stained cross sections (Fig. 4a, 4b, and 4c) the number of *LacZ*-positive cells observed on days 7, 28, and 92 posttreatment was maintained at similar levels (Fig. 2b) confirming the persistence of expression produced by our LV-mediated gene transfer protocol.

It is important that the appropriate airway epithelial cell(s) are transduced when developing gene transfer protocols for CF gene therapy (Parsons *et al.*, 2000a). *LacZ*-positive cells were observed

in all regions of the nasal airway with the exception of the olfactory and squamous regions. Quantitative determination of *LacZ*-positive cells was restricted to areas of respiratory and transitional epithelium, which both contain ciliated cells. The types of transduced cells were determined in H&E-stained sections. We found that transduced cells were predominantly ciliated and nonciliated; smaller numbers of transduced secretory (predominantly goblet) and basal cells were also seen. The numbers of each cell type showed significant changes over the duration of the experiment (Fig. 5). Of note was that *LacZ*-positive secretory cells were only observed at 92 days posttreatment.

Correction of the CFTR defect in the cfr^{tm1Unc} mice

Six cfr^{tm1Unc} mice were exposed to 1% LPC 1 hr prior to the instillation of the LVCFTR vector (4×10^4 NIH3T3-TU). As a control for the effect of LPC alone, three cfr^{tm1Unc} mice were exposed to 1% LPC 1 hr prior to the instillation of carrier solution (PBS).

Decreasing (more negative) Δ PD values reflect increasing functional correction of defective CFTR-mediated chloride secretion (Parsons *et al.*, 1998). Untreated cfr^{tm1Unc} mice exhibited a Δ PD value of $+9.5 \pm 1.2$ mV compared to heterozygote CF colony mice (-16.5 ± 2.0 mV). Treatment of homozygote cfr^{tm1Unc} mice with LPC prior to PBS (control) instillation did not alter the Δ PD value when examined 7 days later ($+8 \pm 3.2$ mV).

Seven days after exposure to the LVCFTR vector functional expression of CFTR in cfr^{tm1Unc} mice resulted in a more negative (but not significantly different) mean Δ PD value ($+2.5 \pm 2.2$ mV) compared to that of untreated cfr^{tm1Unc} mice (Fig. 6a). Forty-six days posttreatment functional recovery of CFTR activity had improved 54% towards the mean heterozygote Δ PD value, a statistically significant improvement (Δ PD = $-4.5 \pm$

3.1 mV, $p < 0.05$ ANOVA, Dunnet multiple comparison) from the (control) untreated CF mouse Δ PD value. At 110 days after LVCFTF vector instillation partial functional CFTR correction had persisted in one of the two surviving LV-treated *cfr^{tm1Unc}* mice (Δ PD = -1.7 mV), while in the second mouse the Δ PD had waned (Δ PD = +5.5 mV). By 13 months the Δ PD values in both these mice had declined further (Δ PD = +5.0 mV and Δ PD = +6.0 mV; 17% and 13% of mean heterozygote Δ PD values, respectively).

The effect of the LVCFTF vector instillation protocol on the basal TPD values is shown in Figure 6b. The apparent reductions in TPD present at 7 and 46 days did not reach statistical significance.

DISCUSSION

For gene therapy to evolve as a suitable treatment for CF lung disease ways of improving the historically low efficiency of gene transfer to airway epithelium must be found. Modulating the airway epithelium to enhance gene transfer, for example by altering TJ permeability to improve access of vectors to their receptors on basolateral surfaces, is an approach that has only recently received attention. Improving basolateral access should also allow enhanced gene transfer to subapical progenitor cells (Engelhardt, 2001). Successful transduction of progenitor cells would be expected to result in the generation of a stable population of gene corrected airway epithelial cells over long time frames. Although airway progenitor cells have recently been identified in the rodent trachea (Borthwick *et al.*, 2001), the identity of progenitor cells in human ciliated airway epithelium remains controversial.

Increased efficiency of gene transfer after modulation of airway TJ has already been demonstrated. For example, treatment with ethyleneglycoltetraacetic acid (EGTA) (a Ca^{2+} chelator) in a hypotonic buffer opens epithelial TJ and improves the efficiency of *in vivo* gene transfer provided by vectors based on feline immunodeficiency virus (Wang *et al.*, 1999) adenovirus (AdV) (Wang *et al.*, 2000; Chu *et al.*, 2001), and retrovirus (Wang *et al.*, 2000) vectors. In contrast to the relatively slow

action of EGTA (1 hr to achieve opening of TJ), apical application of sodium caprate on well-differentiated airway epithelial cells *in vitro* resulted in the rapid opening of the airway epithelial TJ, also improving AdV-mediated gene transfer (Coyne *et al.*, 2000). In addition, exposure of the mouse nasal epithelium to the toxic gas SO_2 prior to the instillation of a VSV-G pseudotyped LV vector expressing the *LacZ* marker gene improved the efficiency of LV-mediated gene transfer *in vivo* (Johnson *et al.*, 2000).

Our modifications to the detergent-based procedures originally developed for the AdV vector (Parsons *et al.*, 1998) have shown that pretreatment of mouse nasal airway epithelium with LPC considerably enhances the capability of our LV vector to transduce intact airway epithelium. The key finding of this study was that exposure to a single dose of a LV vector carrying the CFTR gene could produce extended (at least 110 days) electrophysiologic recovery of CFTR function in *cfr^{tm1Unc}* mouse nasal airway. Because gene transfer was not observed unless LPC conditioning was used we believe the role and use of LPC (or other surface-active agents) for airway surface conditioning warrants more detailed investigation and development.

LPC is the detergent component of pulmonary surfactant (comprising 2%–5% of total phospholipids) (Weltzien, 1979; Niewoehner *et al.*, 1989). Apart from the TJ modulation effects of LPC (Parsons *et al.*, 1999) there are several potential mechanisms by which LPC could modulate the airway epithelium. The mucolytic properties of LPC could solubilize airway mucus by reducing its viscosity and elasticity (Martin *et al.*, 1978); LPC should also reduce MCC activity because it is able to reduce ciliary beat (Merkus *et al.*, 1993); indeed, we have observed dose-dependent reversibility of reduction in ciliary beat in freshly excised mouse nasal airway epithelium (M. Limberis, unpublished data). In pilot studies LPC has improved surrogate vector particle deposition onto airway epithelium *in vivo* (Parsons *et al.*, 2000b). While there are likely to be other biologic effects of LPC (Prokazova *et al.*, 1998) that may be relevant to gene transfer, the direct airway surface effects described here would each be expected to contribute to enhanced gene transfer *in vivo* by improving both retention of gene transfer vector particles after deposition, and subsequent access to the basolateral cell surface.

FIG. 3. *LacZ* gene transfer after a single delivery of LVLacZb into the lysophosphatidylcholine (LPC) pretreated mouse right nasal airway. **A:** Seven days. *En face* anteriorly directed view of septum (s) and turbinates (nasoturbinate (nt), maxilloturbinate (mt)) at 7 days. The undosed (left) nasal airway displays no *LacZ*-positive cells, or regions, while the treated (right) side shows scattered *LacZ*-positive cells along the vertical face of the septum and in some faces of the nasal turbinates. The thick arrow shows direction of view of septum face in (C) and (E). Note lack of *LacZ*-positive cells in the untreated nasal airway. Section is similar to that of Level 16 (Mery *et al.*, 1994), where extensive detail on mouse nasal airway anatomy and cell types is available. **B:** Seven days. Situated below the brain (br) at the posterior of the nasal cavity, moderate cell staining is present in the nasopharyngeal meatus on only the ipsilateral (right) portion of this airway, corresponding to the dosed nostril (arrow). **C:** Twenty-eight days. View of the septum wall as indicated by arrow in (A). The remaining nasal airway of the ipsilateral (dosed) nostril has been cut away to allow this view. The patchy punctate blue staining of *LacZ*-positive cells that contrasts with the diffuse blue-green background stain characteristic of X-gal processing in unsectioned tissue (here, in the olfactory region) is apparent. **D:** Twenty-eight days. *LacZ*-positive cells in nasopharyngeal meatus (arrow) again remain ipsilateral. **E:** Ninety-two days. View of the X-gal-processed septum and left nostril (the curve of the contralateral dorsal olfactory region is visible here outlined by the diffuse background X-gal stain). Scale bar applies to (A), (C), and (E). **F:** Ninety-two days. Strong and extensive *LacZ*-positive cell staining present in the ipsilateral half of the nasopharyngeal meatus (arrow) reveals persistence of *LacZ* expression for 3 months after the single dose of the LV vector. Scale bar applies to (B), (D), and (F).

FIG. 4. Details of anterior nasal *LacZ* gene expression after lentivirus (LV)-mediated gene transfer. *LacZ* gene transfer into ciliated airway epithelium on the nasal septum was limited to the treated right nostril. Safranin O (Saf-O)-stained sections at (a) 7 days, (b) 28 days, and (c) 92 days after exposure to the LVLacZ vector show gene expression as individual dark blue-stained cells, or groups of *LacZ* positive cells (arrows).

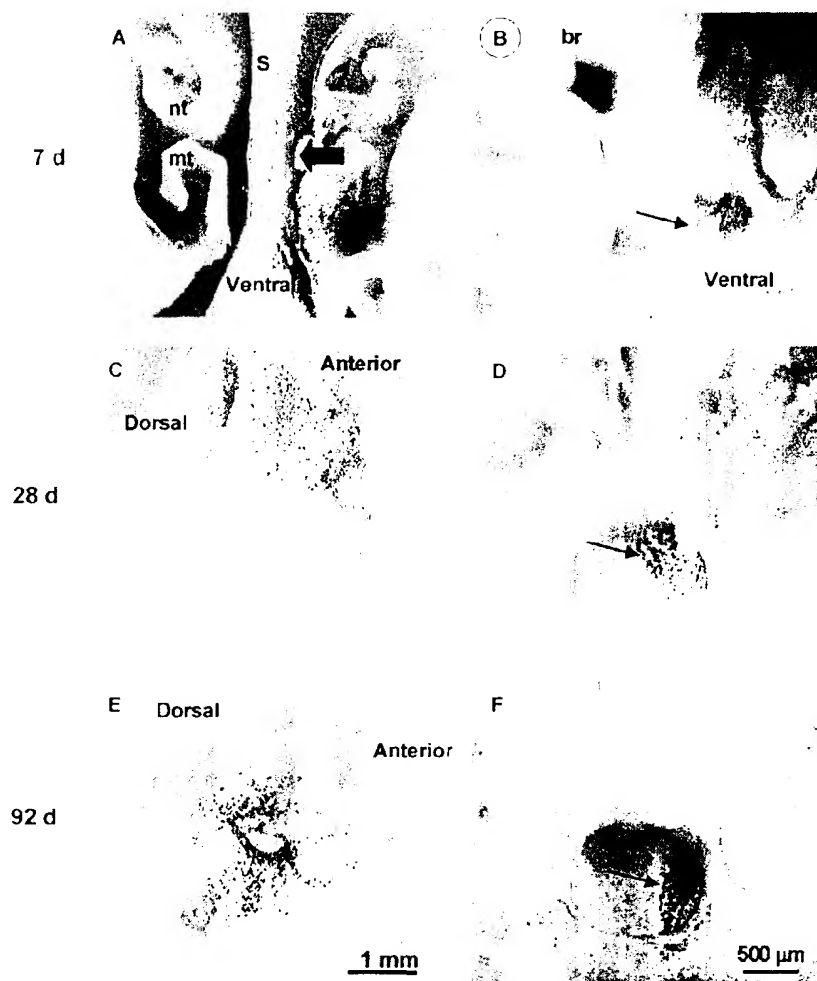


FIG. 3.

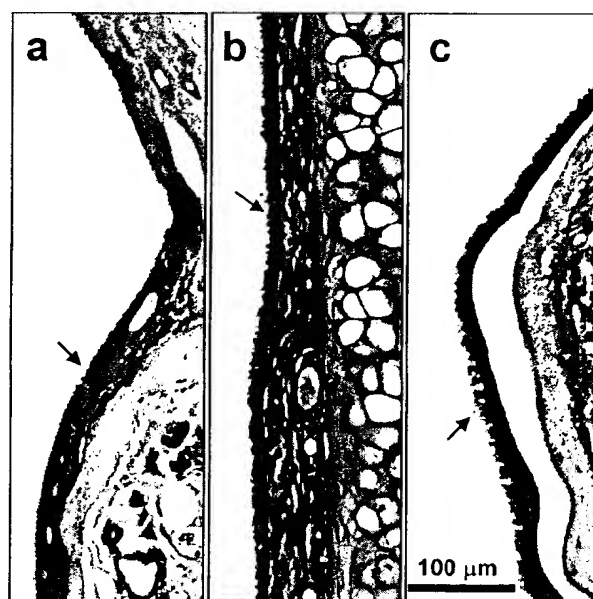


FIG. 4.

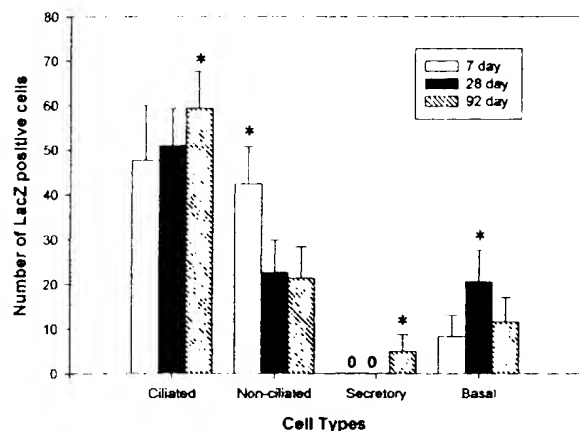


FIG. 5. Types of *LacZ*-positive cells in mouse nasal airway. The significance of changes in the proportion of transduced cells of each cell type over the three assessment time points was individually examined using logistic regression analysis. For each cell type the proportion of transduced cells altered significantly during the assessment period (*Ciliated $p = 0.01$; nonciliated $p < 0.001$; secretory $p < 0.001$; basal $p < 0.001$). Statistically, the significance of the results for the secretory cells should be regarded as approximate, given the zero counts on days 7 and 28.

Ideally, a pretreatment/conditioning agent should produce a transient and tolerable perturbation of the barrier function(s) of ciliated airway epithelium. Histologic injury has been observed immediately after dosing with 1% LPC alone (i.e. limited areas of deciliation or exfoliation found anteriorly close to the dosed site; D.W. Parsons, unpublished data), and this same LPC exposure prior to LV vector instillation was associated with effective *LacZ*, or CFTR gene transduction (Fig. 6, 2a and 2b). However, we also found persisting *LacZ* gene expression in nasal epithelium as far posterior as the nasopharyngeal meatus (Fig. 3B, 3D, and 3F) a site where LPC-induced cell injury was not observed. It thus appears the more dilute concentrations of LPC reaching this region also permit LV-mediated gene transfer. This finding is consistent with the data showing that milder (noninjurious) LPC-based airway modulation is also effective in enhancing the effectiveness of other gene transfer vectors (Parsons *et al.*, 1999). Endogenous LPC is rapidly converted in cell systems (Besterman and Domanico, 1992) and lung alveoli (Seidner *et al.*, 1988) to the ubiquitous and nontoxic dipalmitoylphosphatidylcholine (DPPC), a primary component of biologic membranes. We speculate that there is, therefore, some capacity for exogenous LPC to be similarly converted *in vivo*, providing a measure of active removal that is not a feature of other airway barrier modulation reagents reported to date.

Interestingly, we found that the widely used enhancer for retroviral vectors, Polybrene (Coelen *et al.*, 1983), appears to inhibit LV-mediated gene transfer when used *in vivo*. This finding highlights the need to question assumptions generated from *in vitro* experiments when progressing to *in vivo* trials.

Because permanent expression of functional CFTR in CF airways is the primary goal in efforts to develop a cure for CF lung disease, the greater than three-month persistence of LV-mediated *LacZ* gene expression we found after a single vector administration was encouraging. The number of *LacZ*-positive

cells showed an increase (nonsignificant) at 92 days (Fig. 2b). As the cell turnover time of rodent airway epithelium is thought to be in the order of approximately 3 months (Borthwick *et al.*, 2001), the total number of *LacZ*-positive cells observed should have dropped substantially by 92 days unless airway progeni-

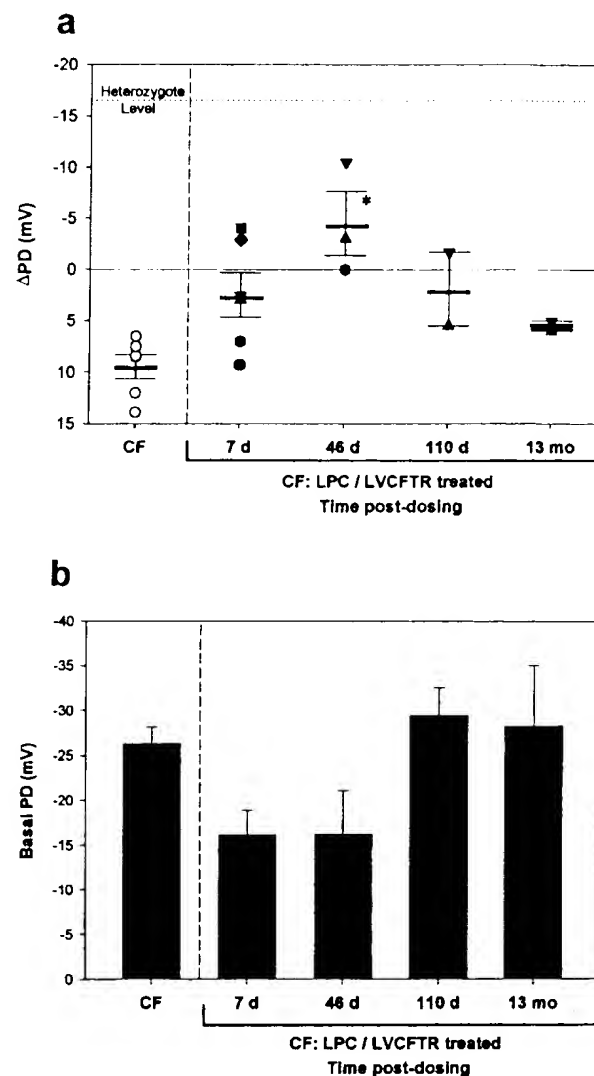


FIG. 6. Effect of LVCFTF administration on nasal airway Δ PD. **a:** Between 7 days and 13 months the mean Δ PD (horizontal bars: mean with standard error [SE]) as well as individual (time-linked) Δ PD values (symbols ■, ◆, ▼, ▲, ●, ●) are shown. Significant partial correction of cystic fibrosis transmembrane conductance regulator (CFTR) electrophysiologic function compared to the mean Δ PD of untreated CF mice ("CF", $n = 6$) was present at 46 days (* $p < 0.05$, analysis of variance [ANOVA], Dunnett multiple comparison, $n = 3$). At 110 days the Δ PD of one mouse remained high, but by 13 months the Δ PD for both remaining mice in this study had waned to near-untreated CF mouse Δ PD mean values. **b:** Basal TPD of nasal airway after LVCFTF dosing protocol. ANOVA analysis indicated that there was a significant difference between the five treatment groups ($p = 0.03$) but subsequent multiple comparisons against the untreated CF control group (Dunnett's method) did not identify the source of the significant TPD reduction(s). Power (0.58) was below that required in this study (0.80).

tor cells had been transduced. In support of the belief that progenitor cells were transduced, we noted that although *LacZ*-positive secretory cells were not seen at 7 or 28 days, they were present at 92 days (Fig. 5). This suggests that outgrowth and differentiation of (transduced) progenitor cells into secretory cells may have occurred between 28 and 92 days. Given the current rudimentary understanding of stem cell identity and physiology in airway epithelium the reason for the changes in the numbers of *LacZ*-positive ciliated, nonciliated and basal cells across the three assessment time points cannot be addressed here. Presumably, the changes observed represent a dynamic balance between turnover of mature cells, and their replacement by outgrowth and differentiation of various progenitor populations, each of which will display a different initial transduction efficiency.

We did not specifically examine the effect of our LVCFTF gene transfer protocols on the sodium hyperabsorption that is characteristic of CF airway dysfunction (via comparison of airway PD values in amiloride-supplemented/free perfusion solutions). Nevertheless, the (nonsignificant) reductions in mean basal PD value (an index of sodium channel activity) apparent 7 and 46 days after LVCFTF vector instillation suggested the LVCFTF dosing protocol might be able to alter the basal TPD. However, the values present at the later time points are clearly no different to the mean basal TPD values present in untreated CF mice. Additional studies that include appropriate TPD comparisons using amiloride-supplemented solutions are indicated, both to improve statistical power and to directly examine how CF airway sodium hyperabsorption is altered by LVCFTF vector exposure.

Correction of the electrophysiologic defect in the Δ PD value of CF mice by LVCFTF gene transfer appears to have begun to decline by 110 days in this study. Several factors may contribute to this apparent difference in persistence in expression of the *LacZ* and CFTR genes. First, the measurement of nasal TPD in mice has inherent technical limitations (Parsons *et al.*, 2000a) and we believe that such limitations may contribute to the variability observed in TPD values at each assessment time point. In particular, at each TPD assessment the cannula tip may not sample from precisely the same area of airway epithelium. The complexity of the mouse anterior nasal anatomy (Parsons *et al.*, 2000a), the relative positional changes in nasal anatomy that accompany growth, and the variability inherent in nasal cannula insertion procedures performed many weeks or months apart mean that larger numbers of mice will be needed to overcome this source of variability in any studies using reassessment of functional CFTR gene expression in nasal airway over time. Second, differences in the completeness of sampling of gene expression may be important. *LacZ* gene expression provides a visible and unambiguous assessment applicable to both the entire nasal airway (Fig. 3) and to standard samples of airway (Fig. 4), whilst measurement of CFTR gene expression samples a restricted area of airway epithelium under the TPD cannula tip.

Nevertheless, the partial correction of the electrophysiologic defect resulting from LV-mediated delivery of the CFTR gene in CF mice diminished between 46 and 110 days in this study, and had almost entirely disappeared by 13 months. The reasons for the discrepancy in gene expression persistence produced by the LVLacZ and LVCFTF vectors is not known; however, we note that neither the cell types requiring CFTR correction, nor the cells that produce the electrophysiologically measured

changes in epithelial TPD, are described for intact airway. The link between the Δ PD value, the level of CFTR expression per cell, and the percentage of cells expressing vector-delivered CFTR is also unknown *in vivo*. Understanding these relationships should provide key information to help direct the development of more efficient airway gene vectors; clearly, longer term detailed studies using both the *LacZ* marker gene and the CFTR gene will be required to resolve these issues.

Because CF lung disease takes many years to establish, and because it is resistant to current therapies and often includes pathologies not directly related to CFTR dysfunction (e.g., airway wall damage subsequent to chronic bacterial infection), recovery of CFTR function alone is unlikely to produce immediate restoration of lung function in already diseased lungs. Gene therapy for CF lung disease will therefore be targeted to the early childhood period prior to the acquisition of lung infection. Before this approach could be considered, parents and patients with CF, researchers, and clinicians must be satisfied with the safety profile of both the gene transfer vector(s) and of any airway-conditioning reagent(s) used. However, our demonstration of persisting *in vivo* CFTR gene transfer after simple dosing procedures, and the recent developments in targeting and potential dosing simplicity provided by novel LV envelope pseudotypes (Kobinger *et al.*, 2001) offer hope that the promise of LV gene therapy (Friedmann, 2000) can indeed be translated into a safe and effective treatment of CF lung disease. The simplicity of our transduction protocol, which utilizes brief single exposures to LPC and LV vector, should facilitate further development toward clinical applications.

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Status of gene therapy for cystic fibrosis lung disease

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Perspective

SERIES
on cystic fibrosis

James M. Wilson, Editor

*This series continues from
the February 1999, no. 3,
issue. See also pages
447-452 in this issue.*

Gene therapy for the treatment of cystic fibrosis should be a "natural" Cystic fibrosis (CF) is a recessive disease associated with loss of function mutations in the CF transmembrane conductance regulator (CFTR) gene, which has a well characterized gene product; heterozygotes, as predicted, appear to be phenotypically perfectly normal; the level of expression of CFTR in affected cells generally appears to be low; and the dysfunctional epithelial lining cells in the organ most affected by CF (the lung) are available for direct vector delivery via topical administration (1). However, despite an impressive amount of research in this area, there is little evidence to suggest that an effective gene-transfer approach for the treatment of CF lung disease is imminent. The inability to produce such a therapy reflects in part the learning curve with respect to vector technology and the failure to appreciate the capacity of the airway epithelial cells to defend themselves against the penetration by moieties, including gene-therapy vectors, from the outside world. This Perspective will focus on the issues that impact on moving this field forward.

What is the target for CF gene therapy in the lung? Cystic fibrosis affects the conducting airways of the lung and not the alveolar surfaces. The airways in general consist of a "large" airway (bronchial) region that is lined by a pseudostratified columnar superficial epithelium and contains numerous submucosal glands, and a "small" airway (bronchiolar) region that is lined by a simple columnar epithelium and is devoid of glands. Central issues for CF gene therapy are which region (large vs. small) and which tissue (superficial epithelium vs. glands) should be targeted.

Obviously, the answer to this question requires knowledge of the pathogenesis of CF lung disease. As reviewed earlier in this Perspective series (2, 3), this is a controversial issue. Although the so-called "isotonic" and "hypotonic" airway surface liquid theories have different predictions on the pathogenesis of CF airways infection, both agree that defects in the superficial epithelium may initiate CF lung disease. However, studies from other model cell culture systems, like Calu-3 cells and cultured gland acini (4), predict that there may be abnormalities in gland volume/compositional (HCO_3^-) regulation in CF that may be more important in the pathogenesis of CF airways infection. This debate can also be viewed in the context of the individual cell types in the airways. Advocates of the importance of the superficial epithelium in CF pathogenesis likely would favor targeting the ciliated cell, which clearly exhibits all of the ion transport functions of CFTR and exhibits abnormal

function in patients with CF (5), whereas advocates of the importance of the submucosal gland would likely favor targeting the submucosal gland serous cells, which may be the highest CFTR-expressing cell type in the lung (6).

In the absence of definitive data from model systems, from an operational point of view probably the best strategy is to examine the sequence of disease in young CF patients and select the target based on those data. Perhaps the most relevant observations are that CF infants typically present clinically with physical and roentgenographic findings of bronchiolitis, exhibit as their first pulmonary function abnormality small airways obstruction, and have evidence from autopsy studies of mucus plugs in small airways. These data suggest that as in other major airway diseases — chronic bronchitis, for example — small airways are the initial and major site of functional disease (airflow obstruction) in the CF lung. Therefore, restoration of function in the superficial epithelium lining small airways should be clinically beneficial. This reasoning does not dismiss expression of abnormal function in proximal CF airways. Indeed, virtually all studies of epithelial dysfunction in the lung have detected differences in this region, but the importance of small airways obstruction in the phenotype of airways disease suggests that selective correction of epithelial defects in the large airways will not be therapeutically useful. Interestingly, virtually all gene-therapy trials to date have delivered vectors via the topical route to the superficial epithelium, but it is not obvious that aerosol delivery strategies have been optimized for small airway deposition. Although deposition is difficult in patients with airways occluded by mucus plugs and infection, it will be important to develop efficient means to deliver vectors via aerosol to small airways.

However, it is possible that it may be important to treat submucosal glands and that we will not be able to devise strategies to effectively dose CF airways. Therefore, it would appear prudent to continue efforts to deliver vectors systemically that could access gland regions as well as the superficial epithelium of occluded airways.

How much gene transfer is enough? A key issue is to distinguish between the concepts of "level of CFTR transduced/cell" and "percent correction," denoting the fraction (percentage) of CF cells within an epithelial region (area) that are "corrected." With regard to level of transduced CFTR/cell, based on endogenous CFTR expression data it is likely that the level required for ciliated cells will be very low whereas the level required for

serous cells will be higher (4). With regard to percent correction, initial studies focused on this issue utilized monolayers of immortalized CF epithelial cells comprised of varying percentages of CF cells and CF cells "corrected" with wild-type CFTR (7). These studies showed that approximately 6%-10% of the cells within a monolayer must consist of "corrected" CF cells to restore normal Cl⁻ transport function.

While informative, the study by Johnson *et al.* emphasizes the importance of both knowledge of the pathogenesis of CF lung disease and the fidelity of the model system to the *in vivo* situation to accurately address this issue. For example, the epithelial model system used for these studies comprised a mosaic monolayer epithelium that was highly connected via gap junctions, utilized "corrected" CF cells that expressed rather high levels of CFTR per cell, and focused only on Cl⁻ transport. The "amplification of correction" (*i.e.*, normalization of function with correction of a small percentage of epithelial cells) reported in that study likely reflected the movement of Cl⁻ ions from non-corrected to corrected cells through gap junctions, with Cl⁻ secretion reflecting exit through a "lot" of CFTR in a small number of corrected cells. It is likely that the number of gap junctions per cell in a well-differentiated epithelium *in vivo* is less than in the immortalized airway cells used in this study, and hence the percentage of cells requiring correction to restore normal Cl⁻ transport *in vivo* may well exceed 10%.

The relationship between normalization of function and percentage of corrected cells within the epithelium is also quite different if one considers Na⁺ transport. Recent data have suggested that lack of CFTR regulation of Na⁺ transport rates may be important in the pathogenesis of CF lung disease and that the relationship between CFTR and Na⁺ transport is more "local," *i.e.*, may involve protein-protein interactions confined to single cells (1). Thus, when abnormal CF Na⁺ transport is used as an index of correction, one finds a linear relationship between the percentage of cells in a monolayer corrected and the percent normalization of function (8).

Consequently, the simplest strategy to assure efficacy is to mimic the normal pattern of expression, *i.e.*, there should be a low level of expression per cell, and virtually every affected cell (100%) should be corrected. Is there an acceptable percentage below 100% of cells that might justify the initiation of a clinical trial? Given the likely difficulties in achieving gene transfer in man *in vivo* compared to any model system, certainly more than 10% of cells should be transduced in the most relevant model systems, *e.g.*, studies of human explants and pertinent animal models *in*

vivo. Unfortunately, none of the current *in vivo* model systems, such as the CF mouse, yield a sufficiently accurate lung infection phenotype to allow this critical question to be evaluated in a whole animal system.

Where are we in the clinic? Approximately 20 trials of CF gene therapy dosing the lung have been completed. These studies essentially have all been Phase I safety studies that have delivered both viral and nonviral vectors topically to the nose and/or lower airways via direct liquid instillation or via aerosol. With respect to adenoviral vectors, both single and multiple dosing studies have been performed.

From these Phase I trials, there has been a wealth of data produced on the safety aspects of first-generation nonviral and viral vectors. In brief, there have been no instances of identification and/or recovery of recombinant viruses from viral vectors, and relatively few if any DNA/vector-specific systemic effects resulting from intrapulmonary vector instillation have been detected. There have been reports of both inflammatory adverse events and immunologic responses to vectors. With respect to acute inflammatory responses, tachykinin-mediated neuroinflammatory responses in the nasal cavity in response to high-dose adenoviral vectors have been reported. A syndrome associated with acute pulmonary inflammation has also been reported (9). It is not clear what the etiology of this latter syndrome may be and whether it reflects, in part, deposition of vectors on alveolar versus airway surfaces, and/or the immune status of the patients. An acute, presumably cytokine-mediated response to liposome-mediated gene transfer in the lung has also been reported (E. Alton, personal communication). With respect to more delayed immunologic responses, rather small increases in adenoviral neutralizing antibody titers have been reported without an adverse clinical outcome (10). Although the data are more sparse, few or no inflammatory/immunologic responses have been reported with the AAV vectors.

With respect to gene-transfer efficiency/efficacy, perhaps the most quantitative data available are from studies that have dosed the nasal epithelium. For adenoviral vectors, initial reports from unblinded studies using nasal PD protocols that discriminated poorly between the CF versus normal phenotype indicated functional correction of CF epithelial Cl⁻ transport (11). Data from larger, placebo-controlled and blinded studies indicate

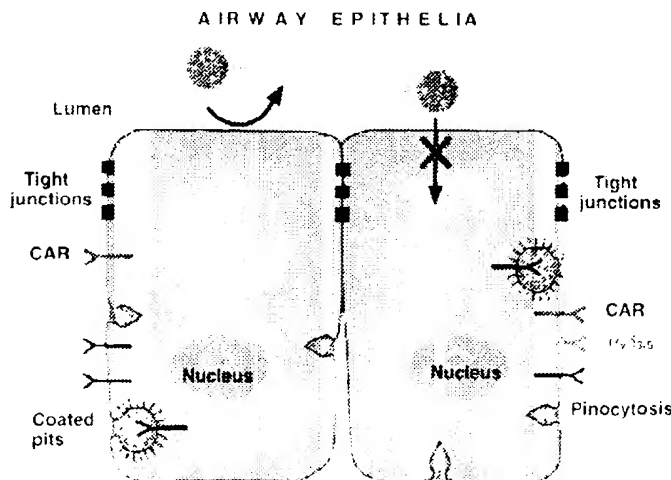


Figure 1

Barriers to vector-mediated gene transfer in WD columnar airway epithelial cells. The failure of vectors to bind to the apical membrane of WD cells is depicted on the left cell; the failure of "non-specifically" bound vectors to internalize is shown on the right cell. The tight junctions separate the apical cell membrane from the basolateral domain that selectively expresses specific viral receptors, *e.g.*, the CAR, "housekeeping"/growth receptors, and integrins. WD, well-differentiated. CAR, coxsackievirus and adenovirus receptor.

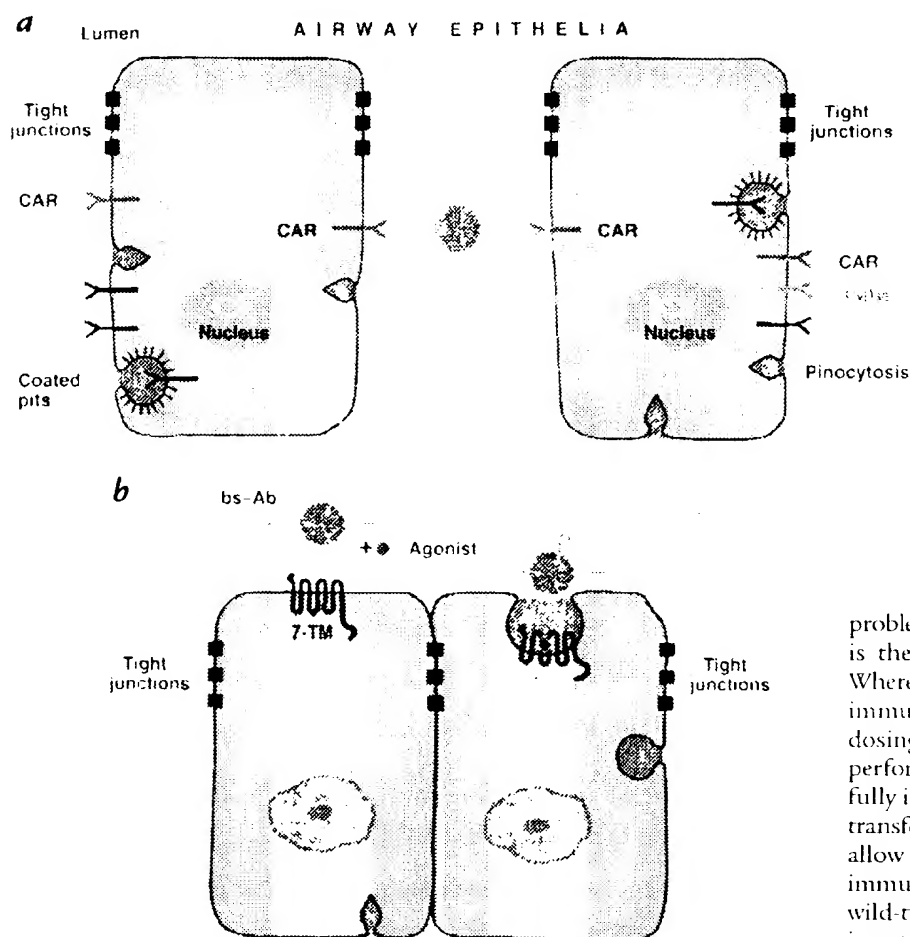


Figure 2

Strategies to increase adenovirus-mediated gene-transfer efficiency for well-differentiated columnar airway epithelial cells. (a) "Modification of the host." In this approach, the tight junctions are rendered permeable to vectors, which permits access to "vector-specific" receptors. (b) "Modification of the vector." A representative seven transmembrane (7-TM) G-protein-receptor is shown that is activated to internalize via interaction with an agonist (denoted by purple-filled circle). Representative (adenovirus) vector is directed toward an external epitope of the receptor by bispecific antibodies (bs-Ab). Upon exposure to agonist, the 7-TM receptor is sequestered into a clathrin coated pit, carrying the vector into the cell.

that topical delivery of adenoviral vector to the nasal epithelium results in little gene transfer or functional correction, as measured with a combination of molecular (PCR) and functional (nasal PD) techniques (12). Similarly, there is little evidence of significant gene transfer with liposome-mediated gene delivery in the nasal cavity, using a variety of lipids and plasmid systems. The data with AAV in the nose are preliminary but also suggest poor efficacy.

Efficacy studies in the lower airways are more difficult to perform because of the difficulty in defining the precise sites of vector delivery and the inability to assess gene transfer quantitatively. With respect to adenoviral gene transfer, PCR assessment of gene transfer has detected wild type CFTR transcripts in brushings from dosed CF airways but there are few quantitative data measuring the percent transduced epithelial cells in the region and no functional (PD) measurements of correction. With respect to liposomes, one nicely designed and performed study attempted to measure functional and molecular correlates of CFTR expression after aerosolized liposome-plasmid dosing of the lung. These investigators reported perplexing evidence for modest correction of Cl⁻ transport, but not Na⁺ transport function in the lung, without molecular (PCR) evidence of gene transfer (E. Alton, personal communication). Finally, although data from AAV administration to the lung are preliminary, they appear to show inefficiency as well.

What is the barrier to successful gene delivery? The major

problem confronting CF gene therapy is the inefficiency of gene transfer. Whereas studies of inflammation and immunologic consequences of vector dosing are important and should be performed, such studies will not be fully informative until adequate gene transfer efficiency is achieved. This will allow the complex inflammatory/immunologic picture of expression of wild-type CFTR in the CF lung to be investigated properly.

Inefficient gene transfer reflects the extremely effective adaptations of airway epithelia to prevent the penetration of foreign materials into airway epithelial cells or the interstitium. Airway epithelia create a complex series of barriers to prevent penetration of lumenally delivered materials, including both viral and non-viral vectors, into the cell or interstitial compartment. In series, these barriers comprise a well-defined mucus layer that may bind inhaled vectors and clear them via mucus clearance mechanisms, a glycocalyx that may bind vectors and prevent binding to cell surface receptors, and perhaps most importantly, an apical cell membrane that is relatively devoid of viral receptors and growth/tropic receptors that internalize as part of their biology (Fig. 1). This series of barriers is complemented by epithelial tight junctions that are "moderately leaky" to ions but quite "tight" for larger solutes, thereby preventing penetration by current vectors from luminal surfaces to the interstitium. Airway cells express most of the receptors that are used by current viral vectors for "virus-specific" entry on the basolateral membrane. Recent reports confirm that specific vector receptors, e.g., the adenovirus receptor (13), the AAV receptor (heparan sulfate) (14), and the VSV receptor (15) are indeed localized to the basolateral membrane. In addition, most of the house-keeping/growth/trophic hormone receptors are also located basolaterally.

The early studies with model systems that employed poorly differentiated airway epithelial cells suggested that gene-transfer efficiency for a variety of vectors

would be high. However, with the advent of the use of well-differentiated (WD) culture systems, supplemented by freshly excised organ culture systems, it became clear that a common theme was emerging: *a*) that virtually all vectors (viral and nonviral) did not bind to the apical (luminal) surfaces of WD airway epithelial cells; and *b*) that apical surfaces of WD airway epithelial cells have a low basal and stimulated rate of endocytosis (13, 16).

What is the answer to increase efficiency? It is apparent that novel strategies must be adopted to increase gene-transfer delivery. As mentioned above, strategies that may use the vascular compartment as a dosing route should be explored but the difficulties in overcoming the large number of barriers between the vascular compartment and airway epithelial cells — endothelial cell, endothelial cell basement membrane, interstitium, and epithelial basement membrane — make this route challenging. With respect to intraluminal dosing of the superficial epithelium, at least two general strategies can be envisioned to increase gene-transfer efficiency.

In a strategy termed "modification of the host," it may be rational to reduce the barrier functions of epithelial tight junctions so that vectors can penetrate to the basolateral membrane of target cells that, as indicated above, are naturally rich in viral and other internalizing receptors (Fig. 2). Abrogation of tight junction barrier function can be achieved by non-specific damage, such as has been demonstrated with oxidant gases (17) and surface-active adjuvants, *e.g.*, detergents (18). Such strategies have been shown to increase gene-transfer efficiency in airways of rodents dramatically, but it likely will be difficult to titrate down the dose of an oxidant gas and/or deliver a specific mass of a detergent safely to make this strategy therapeutic for CF patients. More specific modifications of tight junctional permeability through cellular regulatory mechanisms thus are more appealing. Increasing knowledge of the cellular regulation of tight junctional permeabilities, including the interrelationships between the adherens junction and the tight junction, may make this approach feasible. The ultimate goal is a safe and effective strategy, which depends on: *a*) transient, reversible permeabilization of tight junctions; and *b*) permeabilization of tight junctions without producing inflammation, hence avoiding vascular leak into the airway lumen and airways irritation.

The alternative approach is to "modify the vector." The concept here is to direct a vector to a "target" expressed on the apical cell membrane that has the capacity to both bind and internalize a vector. Identification of suitable targets in the airway has not been easy because of the paucity of expressed receptor/membrane proteins on airway epithelial surfaces that internalize as a function of their biology. However, there is a class of receptors that normally mediates acute airway epithelial cell responses to the luminal environment, *i.e.*, seven transmembrane receptors. Several members of this class are expressed on the lumen of human airway cells.

Perhaps the most attractive target from the point of view of the level and extent of expression in the airways is the extracellular ATP/UTP receptor, termed P2Y₂-R. This receptor internalizes into the cell via clathrin coated pits upon agonist stimulation. Many viruses have evolved mechanisms and escape from clathrin coated vesicles via a

process termed endosomolysis. Preliminary studies in non-polarized cells have documented that either bi-specific monoclonal antibodies directed towards engineered epitopes into the external domain of P2Y₂-R or modifications of the native ligand (BiotinUTP) to direct vectors to P2Y₂-R can produce efficient gene transfer via this pathway (19). More importantly, vector internalization and gene transfer can also be achieved when P2Y₂-R is expressed in the apical membrane of WD cells. Targeting through this approach offers several attractive features, including a wide versatility with respect to the targeting molecules themselves, *e.g.*, antibodies, peptides, or modified ligands, and the ability to link the targeting molecule to a wide variety of vectors, including plasmids, adenoviral, AAV, and lentiviral vectors.

Can we select a preferred vector now? This would be premature. A nonviral vector might be preferable because of the simplicity of the system, but a viral vector, if it were sufficiently safe and efficient, could be a viable alternative. It is likely that we will see a series of both types of vectors developed and used clinically. For example, it is possible that host modification or vector luminal targeting will become a reality relatively rapidly, and that "high-capacity" adenoviral vectors, because of their proven ability to express in airway epithelial cells, their relative safety, and the transient nature of their expression, would be optimal for new studies of safety and efficacy. In the long term, it would appear reasonable that for a genetic disease like CF, integrative gene transfer will be preferable. Thus, it appears wise to continue the development of lentiviral vectors, both HIV (20) and non-human (21), and AAV vectors for this use.

The future. It is clear from analysis of the data describing gene-transfer efficiency from the reported clinical studies that an order or two of magnitude increase in efficiency will be required for gene transfer to be therapeutically relevant in CF. The good news is that all of the previous work has in principle identified the hurdles that must be cleared prior to initiation of novel strategies in man. For example, the WD cultures, freshly excised explant cultures, and bioelectric and expression studies in the mouse nose (but not infection phenotype in the lung) appear to be accurate models for predicting efficacy in man *in vivo*. Further, although there have been questions about its relevance, it does appear that the nose as a first approximation is a good model for lower airways gene transfer in man. Thus, the trial designs in the nose that have been generally agreed upon, *i.e.*, double-blind placebo-controlled studies using nasal PD protocols designed to measure basal Na⁺ transport and Cl⁻ transport, coupled to molecular and morphologic studies with a spectrum of sensitivities, appear to offer a rigorous way to assess the efficacy of a new strategy before initiating more difficult studies in the lung.

A challenge for lung gene transfer, like other forms of CF lung therapy, will be the initial trial design to measure efficacy. Here again, much progress has been made. In the context of exploring drug therapy to treat the initiating cause of disease, trial designs have been explored to assess the ability of novel therapies to protect the lung against disease. Important analyses of the required sample sizes for these studies as a function of patient age have also been recently reported, and healthy discussions on surrogate markers in the lung

are ongoing (22). Thus, one can be optimistic that when we develop strategies that promote routinely between 10% and 100% gene-transfer efficiency in human airways, we will be smart enough not to miss the clinical benefits of gene transfer in CF patients.

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Development of retroviral vectors for gene transfer to airway epithelia

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Retroviral vectors offer several potential advantages for attaining persistent expression of a therapeutic gene in airway epithelia for diseases such as cystic fibrosis. However, several problems have limited their application. Developments in vector production and the advent of lentiviral vectors have increased the investigation of recombinant retrovirus for gene transfer to airway epithelia. In addition, an improved understanding of some of the barriers limiting gene transfer has led to increased transduction efficiencies. The development of novel vector formulations and the use of new envelope pseudotypes are examples of recent findings that are leading to advances in this field.

Keywords Amphotropic, feline immunodeficiency virus, keratinocyte growth factor, lentivirus, pseudotype, retrovirus

Cystic fibrosis - a model disease for the development of airway gene transfer

Cystic fibrosis (CF) is a common autosomal recessive disease with a high carrier frequency among people of Northern European descent [1•]. The gene that is mutated in the disease, the *cystic fibrosis transmembrane conductance regulator* (CFTR), encodes a cAMP-activated chloride channel in epithelia. The CFTR plays an important role in regulating normal electrolyte and liquid transport across epithelia. For most CF patients, chronic lung infection arising from defective pulmonary host defenses gradually destroys the lungs [1•]. The well characterized genetic basis of the disease and the perceived accessibility of the airway epithelium by aerosol delivery or direct instillation has generated significant interest in gene transfer-based approaches to correct CF pulmonary disease.

Within one year of the cloning of CFTR, gene complementation experiments in heterologous cells demonstrated that gene transfer could correct the ion transport defect characteristic of CF [2,3]. Moloney murine leukemia virus (MoMLV)-based retroviral vectors were among the first vectors used to introduce the CFTR cDNA into cells and study its physiological properties [3,4]. Thus, from its inception in the early 1990s, retroviral vectors have played an important role in the development of gene transfer-based approaches to correct this disease. An important issue that must be addressed as any gene transfer-based treatment is developed is the percentage of cells that must be corrected in order to reverse the disease process.

Johnson and colleagues showed in cell mixing experiments that correcting as few as 6 to 10% of the cells within a polarized epithelium generated Cl⁻ transport properties similar to epithelia with 100% corrected cells [5•]. These experiments suggested that for gene transfer to be therapeutic and complement the ion transport defect, it may not be necessary to correct all of the cells in the CF airway epithelium.

In this review we will focus on the development of retroviral vectors, both MoMLV-based and lentivirus-based, for gene transfer to airway epithelia. Before discussing particular vector systems in detail, we will first review the current understanding of some of the barriers to gene transfer that must be overcome in order for this approach to succeed as a therapy for CF.

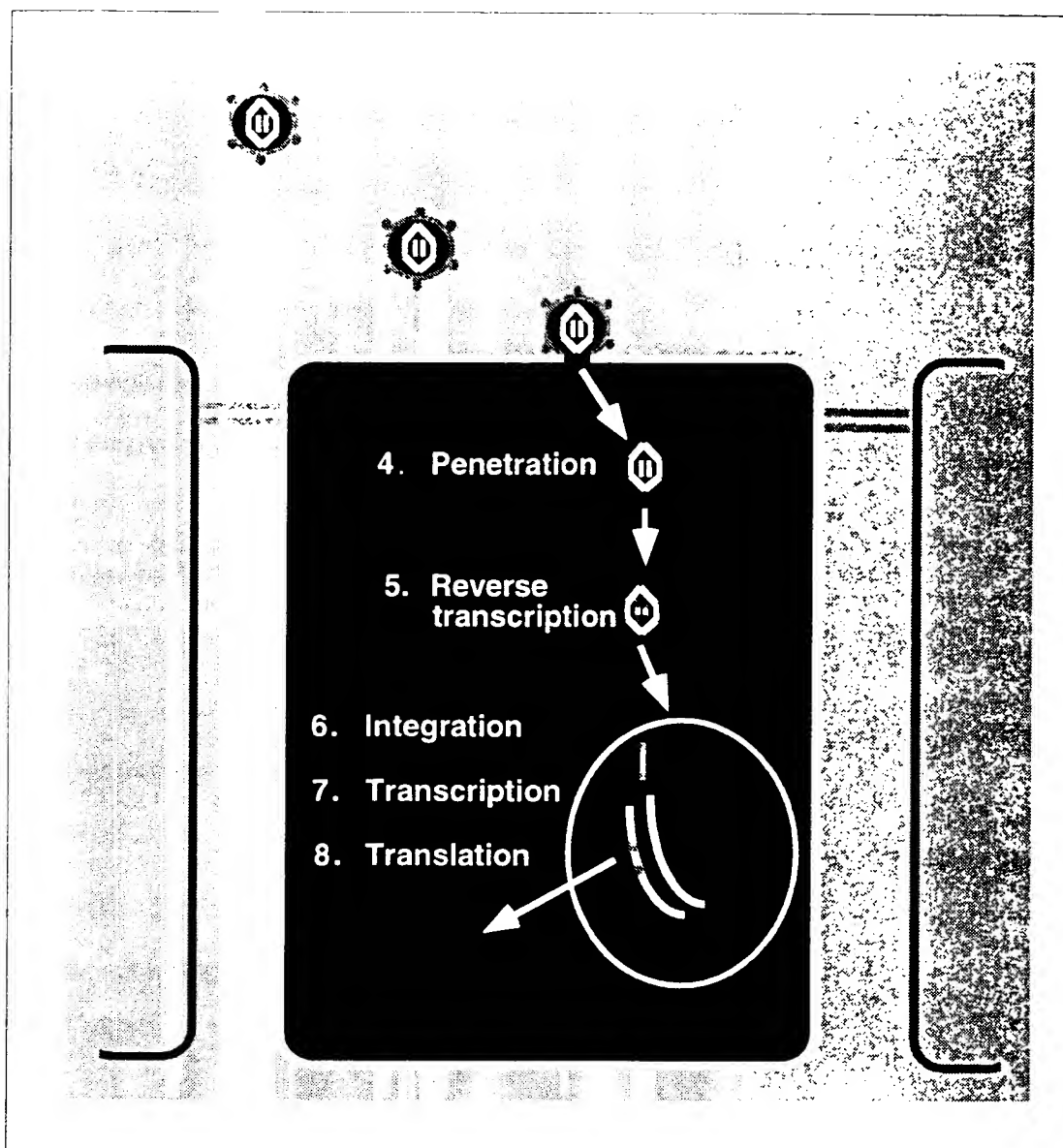
Barriers to gene transfer with retroviral vectors

The airway epithelium possesses several unique properties that make it a formidable target for successful gene transfer. Among these are the many innate and adaptive host defense functions that the epithelium and resident immune effector cells perform [6,7]. The pulmonary epithelium has evolved to prevent the invasion of the host by microbes and these same strategies may act as barriers for gene transfer vectors. Advances in the field of gene transfer to airway epithelia have occurred as our understanding of the cell biology of epithelial host defenses and virus-cell interactions has increased [8•].

To understand the impact of the potential barriers on gene transfer efficiency it is useful to first consider the steps involved in vector transduction. Three steps are required for a retrovirus to enter into target cells, as shown in Figure 1. These are: (i) gaining access to receptors; (ii) binding to receptors; and (iii) fusing with cell or endosomal membranes. The subsequent steps lead to the translocation of the integration complex to the nucleus. For MoMLV or lentivirus vectors, few data exist specifically addressing these issues for airway epithelia.

Multiple factors present on the apical surface of epithelia may act as physical barriers preventing vector access to receptors. These include mucus, airway surface liquid (ASL) and its components, immune effector cells, such as macrophages and neutrophils, and the extracellular matrix. McCray and colleagues reported that human alveolar macrophages inhibited gene transfer to airway epithelia with MoMLV-based vectors *in vitro* [9]. This effect was most apparent with lipopolysaccharide (LPS)-activated macrophages and could be partially inhibited by pretreating these cells with glucocorticoids. Airway mucus (gel layer) is produced by mucous cells (goblet cells) and submucosal glands, with additional modifications made by the surface airway epithelium [6]. It is mainly composed of mucoglycoproteins secreted by mucous cells, glycoproteins from serous cells, and glycocalyx from the epithelium [10].

Figure 1. Model of the airway epithelium showing the steps involved in infection with a recombinant retrovirus.



Each of these steps represents a site where barriers for gene transfer could occur. Tight junctions are indicated as dark gray parallel lines.

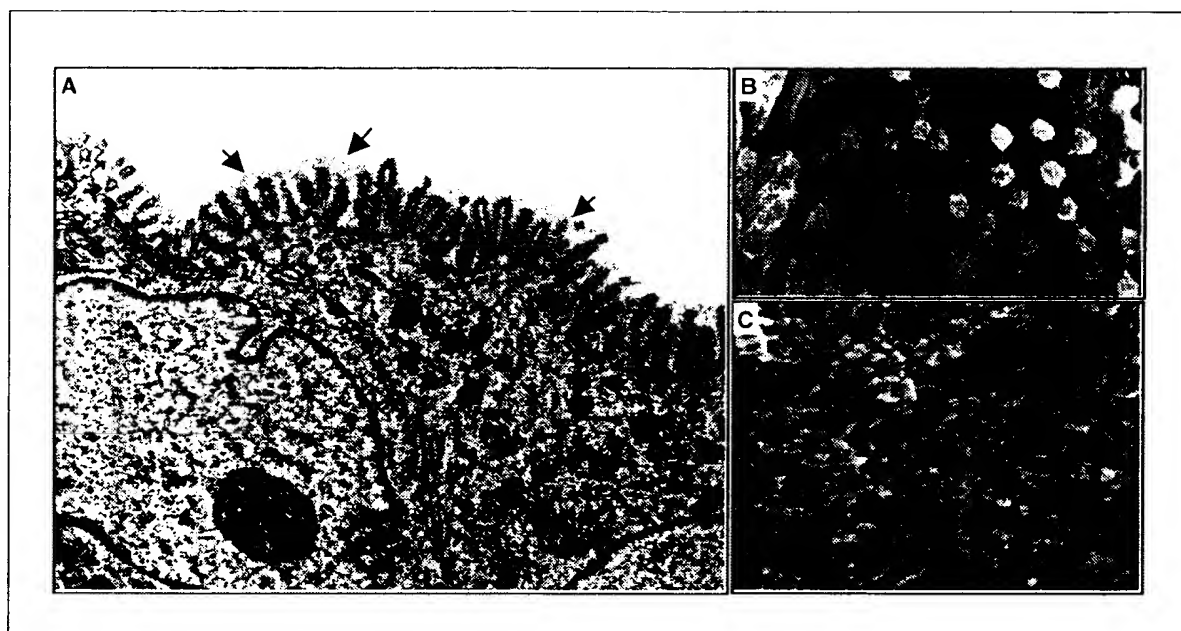
There is evidence that each of these components may inhibit gene transfer to the lung. For example, Kitson and colleagues reported that mucus inhibited liposome-mediated gene transfer to airway epithelia [11]. It remains to be investigated whether mucus inhibits retroviral-mediated gene transfer, but it is likely that excessive airway secretions could act as a physical barrier for gene transfer.

ASL, also known as the sol layer, is defined as a thin layer of liquid (10 to 20 μm) beneath the mucus blanket. In addition to water, the main ASL components are electrolytes, secreted immunoglobulins (primarily IgA), cytokines, antimicrobial peptides and proteins, surfactant proteins, proteinase inhibitors and other epithelial secretions [7]. Several reports have documented that components of ASL play an important role in host defense [7], alterations in its composition may contribute to bacterial colonization and

chronic lung infection in CF [12,13]. Whitsett and coworkers noted that pulmonary surfactant is a potential barrier for gene transfer with both viral and non-viral vectors [14,15], but McCray and colleagues reported that human ASL did not inhibit MoMLV-mediated gene transfer to airway epithelia *in vitro* [9]. However, ASL and its components certainly represent a physical barrier that a gene transfer vector must penetrate *in vivo* to reach receptors.

The extracellular matrix (ECM) is an organized meshwork composed of several locally secreted proteins and polysaccharides that are assembled on the cell surface. Each organ has a unique ECM histoarchitecture that provides cells with biological information and a mechanical scaffold for adhesion and migration. Work has been undertaken to investigate the ultrastructure of the surface of primary cultures of human airway epithelia by transmission electron

Figure 2. Electron micrographs (EM) of the apical surface of primary cultures of human airway epithelia demonstrate a complex glycocalyx.



A. Transmission EM of ciliated airway epithelia. Arrowheads indicate an electron dense matrix. B and C. Scanning EM views of the apical surface of airway epithelia demonstrating a complex matrix material

microscopy (EM) As shown in Figure 2, the apical surface has a layer of ECM meshwork. Although reports have shown that treating the airway apical surface with different proteinases facilitated adenoviral- and AAV-mediated gene transfer [16-18], it remains to be investigated whether ECM constitutes a barrier to retroviral gene transfer to airway epithelia. Likewise, it is not known whether similar treatment of the apical surface of airway epithelia with enzymes would have a positive effect on gene transfer with retroviruses.

Access to receptors is an important step in the process of transduction with any viral vector. For example, the receptors for serotype 2 and 5 adenovirus (Ad) (coxsackie and adenovirus receptor, CAR) and AAV-2 (heparin sulfate proteoglycan) are predominantly expressed on the basolateral surface of airway epithelia [19,20,21]. This finding explains the preferential gene transfer from the basolateral surface of airway epithelia with these vectors. As with Ad and AAV, each retroviral envelope glycoprotein binds to specific proteins on the cell surface to initiate the process of infection. Typically, the protein that serves as a virus receptor performs another function for the cell (Table 1) [22]. Little work has been done to localize the receptors for retroviral envelope glycoproteins in the lung epithelium [15,23].

After the virus binds to its receptor on the cell surface, there is fusion of the virus envelope with the cell membrane or endosomal membranes following endocytosis (Figure 1). This fusion event allows the viral nucleocapsid to enter the cytoplasm where the single stranded RNA is reverse transcribed to DNA and the integration complex is then transported to the nucleus, presumably through interactions

Table 1. Some retroviral envelope glycoproteins and their receptors.

Virus Env pseudotype	Receptor	Reference
Amphotropic (MLV-A)	Pit2 (Ram1, GLVR2)	[63]
Xenotropic (MLV-X)	XPR1	[64]
Ecotropic	CAT1	[65]
GALV (gibbon ape leukemia virus)	Pit1 (GLVR1)	[66]
RD114 (endogenous feline type C virus)	RDR	[67]
10A1 MLV	Pit1 and Pit2	[68]
VSV-G	phosphatidylserine	[69]

with cytoskeletal elements. Due to the complex architecture of polarized epithelia, it is conceivable that barriers could exist at each of these steps that might impede viral entry through the apical surface. This sequence of events has not been investigated in differentiated epithelia cells from the airways.

Approaches to overcome barriers to gene transfer

Several groups are investigating retroviral vectors for gene transfer to the airways, using either MoMLV or lentivirus-based systems. Below we briefly summarize how these vector systems are being developed to overcome some of the barriers to gene transfer.

Rationale for retroviral vectors

In the last ten years a number of viral and non-viral systems have been evaluated as gene transfer vectors for airway epithelia. For the treatment of an inherited chronic disease, such as CF, retrovirus-based vector systems may offer several advantages. These include their ability to integrate

and persist, and an adequate packaging capacity for the CFTR cDNA. Furthermore, the envelopes of retroviral vectors may be less immunogenic than the proteins of encapsidated viral vectors, such as Ad or AAV vectors [24]. However, the early development of retroviral vectors for this application lagged to some degree compared with other systems due to the technical hurdles associated with producing vector of sufficient titer for *in vivo* investigation. Several advances in production, including improved packaging cell lines, novel methods of vector purification and the development of envelope pseudotyping have aided the field [25•,26•,27•].

Murine leukemia virus-based vectors

The ability of retroviral vectors to integrate has led to their widespread use for a number of *ex vivo* and *in vivo* gene transfer applications. Recombinant vectors based on MoMLV were the first retroviruses used for gene transfer to airway epithelia. Engelhardt and colleagues demonstrated that MoMLV-based retroviral vectors transduced airway epithelia *ex vivo* and could reconstitute a fully differentiated epithelium in a tracheal xenograft model [28•]. In a subsequent study, the same group noted that the *in vivo* transduction of epithelia in differentiating grafts only occurred to a significant degree when the cells were proliferating and poorly differentiated [29]. Olsen and coworkers transduced proliferating CF tracheal epithelia using a MoMLV-based vector expressing the CFTR cDNA [30]. When the cells were subsequently allowed to differentiate *in vitro*, they found that the CF transport defect was corrected and CFTR expression persisted for several months [30]. These studies not only succeeded in demonstrating the potential for MoMLV-based vectors to persistently transduce airway epithelia, but also pointed to their requirement for cell proliferation for efficient transduction.

Low mitotic indices of airway epithelia limit gene transfer with MoMLV-based vectors

A striking characteristic of MoMLV-based vectors is their requirement for cell division in order for the integration complex to enter the nucleus [31•]. However, the normal airway epithelium is mitotically quiescent (< 1% of cells dividing) [32•,33•,34]. Halbert *et al* delivered an amphotropic pseudotyped MoMLV vector expressing the human placental alkaline phosphatase gene into rabbit trachea *in vivo*. They reported that transduction efficiency was low and gene transfer only occurred in areas where the trachea was injured [35]. Gene transfer to the airways of developing animals may have a higher efficiency due to increased epithelial proliferation [32•]. Pitt and coworkers took advantage of increased cell proliferation rates in the developing fetal lung, and applied amphotropic MoMLV-based vectors directly into the liquid-filled trachea. However, only limited gene transfer was noted in the airways [36]. Johnson *et al* similarly noticed that adult murine airway epithelia transduced poorly with VSV-G pseudotyped MoMLV-based vectors, and noted some advantage of increased transduction in younger animals [37].

To attempt to overcome the limitations posed by the low mitotic rate in adult animals, Wang and coworkers pretreated neonatal rats with intratracheal keratinocyte

growth factor (KGF) to stimulate airway epithelial cell division [23]. This maneuver resulted in proliferation of ~ 40% of the bronchiolar and alveolar epithelial cells. A high titer amphotropic MoMLV-based vector was then instilled into the KGF-stimulated airway. However, the proliferation indices greatly exceeded the gene transfer efficiency [23]. Similar results were reported by Zsengeller and colleagues in mice [15•]. These studies suggest that barriers other than low cell proliferation rates limited gene transfer with MoMLV-based vectors.

To address this discrepancy between the epithelial proliferation rate and the poor transduction efficiency with MoMLV-based vectors, *in vitro* studies using polarized, differentiated epithelia were performed [38•]. KGF stimulated approximately 50% of human airway epithelia to proliferate. When amphotropic or xenotropic MoMLV vectors were applied to the apical surface of the KGF-stimulated cells, no gene transfer occurred. However, when the same vectors were applied to the basolateral surface, efficient gene transfer was achieved [39•]. Similar findings were noted in cultured rat airway epithelia [23]. Thus, the apical and basolateral surfaces of well differentiated airway epithelial cells have very different viral transduction properties.

Lentivirus-based vectors

Lentiviral vectors infect non-dividing cells, and therefore may offer advantages over MoMLV for achieving persistent gene transfer to the mitotically quiescent airway epithelium [40•]. Several lentiviral vector systems have been developed based on primate viruses (HIV, SIV) [40•,41,42]. Goldman *et al* used a VSV-G pseudotyped HIV-based vector to transduce non-dividing human airway epithelia *in vitro* and in human bronchial xenografts [43•]. The HIV-based vector easily transduced poorly differentiated cells in xenografts leading to the stable expression of CFTR. However, the authors noted that this vector failed to efficiently transduce differentiated airway cells when applied to the luminal surface [43•]. In addition to primate lentiviruses, several groups are investigating the use of non-primate viruses to develop vectors. For example, both equine infectious anemia virus (EIAV) [44•] and feline immunodeficiency virus (FIV) [45•,46•] have been used as backbones for recombinant lentiviral vectors. A theoretical advantage for such vectors is that they are not human pathogens and they share little sequence similarity with HIV. This further reduces the chance of homologous recombination which could result in production of wild-type virus, and may therefore offer additional safety advantages for *in vivo* applications.

Access to retroviral receptors limits gene transfer efficiency

Recent understanding of some of the problems limiting the efficiency of gene transfer has come from more detailed studies of the interactions between retroviral vectors and pulmonary epithelial cells. Firstly, there is evidence that the receptors for some retroviral envelope glycoproteins are not abundantly expressed in the airway epithelium (Table 1). Studies by Zsengeller and colleagues suggest that the mRNA abundance of the amphotropic envelope receptor (Pit2) in the murine airway epithelium is quite low [15•]. In

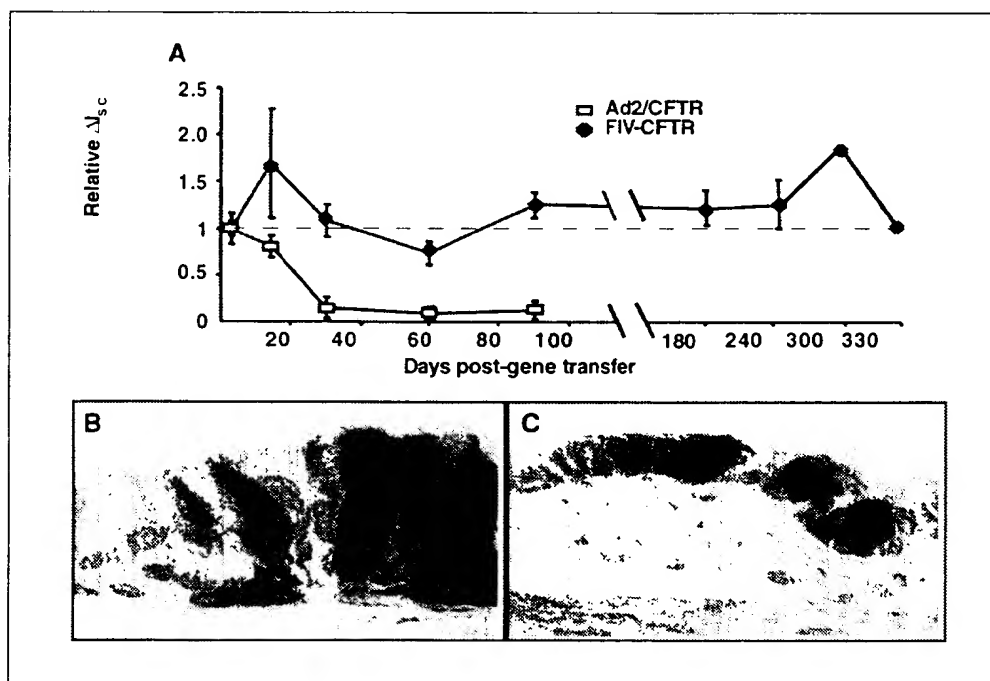
addition to receptor abundance, it has also been shown in human airway epithelia that the receptors for the amphotropic, xenotropic, VSV-G, RD114, 10A1 and GALV retroviral envelopes are not functionally expressed on the apical surface, but are readily accessible if the vector is applied to the basolateral surface [39••, Wang G, McCray P, unpublished data]. These findings confirm the importance of investigating the transduction efficiency of any candidate retroviral envelope pseudotypes in an appropriate polarized epithelial cell model as an early step in developing new vector constructs.

One strategy that has shown early promise for enhancing gene retroviral transfer to polarized cells *in vitro* and *in vivo* is the formulation of vectors in solutions that will enhance receptor access. Wang and colleagues reported that formulating amphotropic, xenotropic or VSV-G-pseudotyped MoMLV-based retrovirus or FIV-based lentiviral vectors in a hypotonic buffer containing 6 to 12 mM *O,O'*-bis(2-amino-ethyl)-ethyleneglycol-*N,N,N,N*-tetraacetic acid (EGTA) greatly enhanced the gene transfer efficiency to human airway epithelia *in vitro* with vectors applied to the apical surface [39••,47••,48•]. Further studies demonstrated that EGTA formulation transiently opened epithelial junctions, allowing vectors access to receptors on the basolateral surface [48•]. Importantly, it was also noted that the FIV-based vector could transduce non-dividing cells [47••]. When the EGTA-formulated FIV vector expressing the CFTR cDNA was applied

apically to primary cultures of well differentiated human CF epithelia, the Cl channel defect was corrected for the life of the culture, a period of 11 months (Figure 3).

Additional studies demonstrated that the rabbit tracheal epithelium and the human nasal epithelium responded to EGTA/hypotonic buffer perfusion *in vivo* with a reversible loss of transepithelial potential and amiloride-sensitive voltage, consistent with the opening of the intercellular junction complexes [48•]. These data suggested that such a vector formulation approach might also enhance gene transfer *in vivo*. Application of the FIV-based vector formulated with EGTA to the luminal surface of rabbit airways *in vivo* transduced ~ 1 to 14% of the epithelia [47••]. Importantly, epithelial cells in the large and small airways were transduced using this approach, and transgene expression persisted (Figure 3) [47••]. Furthermore, cells with apical cytoplasm that does not reach the airway lumen were also transduced using this approach. Similar results were achieved with EGTA-formulated MoMLV-based vectors in the rabbit tracheal epithelium *in vivo* [48•]. Johnson and coworkers recently reported that HIV-based lentivirus pseudotyped with the VSV-G envelope-transduced murine airways poorly *in vivo* unless the animal was first treated with inhalation of sulfur dioxide, presumably to disrupt epithelial integrity and enhance vector access to receptors [49••]. These studies are encouraging

Figure 3. Gene transfer to human airway epithelia with FIV-based lentiviral vectors.



A. Transduction of CF epithelia with an FIV-based vector expressing CFTR *in vitro* leads to persistent correction of the defect in cAMP-activated Cl secretion. ΔI_{sc} indicates the change in short circuit current in response to cAMP agonist stimulation. FIV results are contrasted to those attained with a first generation adenoviral construct expressing CFTR. Results are normalized to measurements at 3 days post-gene transfer. FIV-mediated gene transfer persists while adenoviral-mediated gene transfer is gradually lost.

B and C. Formulating the vector with 12 mM EGTA facilitates *in vivo* gene transfer to the rabbit airway epithelium. Dark cells shown are expressing the β -galactosidase gene in the trachea (B) and smaller bronchi (C).

and suggest that FIV and other lentiviral vector systems might be developed for CF therapies, particularly if the apical barriers limiting gene transfer efficiency are overcome. While additional *in vivo* studies are needed, manipulation of the epithelial junction complex offers one means to overcome poor receptor access with apically applied vectors.

Future directions

Pseudotyping retroviral vectors to target entry via the apical surface of airway epithelia

For *in vivo* applications it is desirable to develop vector systems that can efficiently transduce cells when applied to the apical surface. An attractive alternative strategy to overcome the low efficiency of gene transfer with current envelope constructs is to systematically evaluate glycoproteins from other enveloped viruses for their ability to efficiently infect airway epithelia and use these glycoproteins to pseudotype MoMLV or lentiviral vectors [50••]. Several of the receptors for retroviral envelope pseudotypes have been identified and are listed in Table 1. It is possible that the envelope glycoproteins from some non-retroviral enveloped viruses may be adapted for this purpose. Cosset and coworkers have proven the feasibility of such a strategy by pseudotyping the MoMLV vector with the fowl plague virus hemagglutinin [51]. Chan and colleagues recently reported successful pseudotyping of HIV-based lentivirus vectors using the glycoproteins from Marburg and Ebola viruses [52]. We used a similar strategy to pseudotype FIV-based vectors with the Marburg virus envelope glycoprotein and found that it transduces human airway epithelia *in vitro* when applied to the apical surface [Sinn PL, Wang G, McCray PB, unpublished data]. Several other enveloped respiratory viruses, such as influenza, coronavirus, respiratory syncytial virus and parainfluenza virus, have well characterized envelope proteins involved in binding and fusion that might be adapted for retroviral pseudotyping. Envelope pseudotyping will continue to be an area of very intensive investigation, as it offers the possibility of developing new vector constructs with specific tropism for the apical surface of the airway epithelium.

Development of hybrid and chimeric viral vectors

Over the past few years, investigators have begun to explore the possibility of combining the desirable attributes of diverse families of viruses to maximize their therapeutic potential. One such strategy has the goal of achieving efficient *in vivo* gene delivery and stable transgene expression by developing adenoviral/retroviral hybrid vectors [53-55]. Briefly, replication-deficient adenoviral vectors were designed to express either a reporter/LTR cassette or the MoMLV gag/pol/amphophotropic envelope sequences. These adenoviral vectors were used in concert to infect cells. Following co-infection, the host cell transiently expressed the gene products necessary to package and release MoMLV vectors encoding the reporter gene. Secondary infections occur in neighboring cells, where the integrating capacity of the retrovirus allowed the host cell to persistently express the gene of interest. However, the potential to use such systems to target airway epithelia remains unexplored.

Recently, the advantageous features of Ad and retrovirus were combined to develop a novel hybrid vector system without a requirement for a secondary infection. Zheng and colleagues constructed a replication-deficient recombinant adenoviral vector carrying the 5' and 3' LTR sequences from MoMLV and a *luciferase* reporter gene [56•]. Using this hybrid Ad/MoMLV virus, they demonstrated that luciferase could be expressed in replicating and non-replicating cells *in vitro*, as well as in multiple rat tissues *in vivo*. Of particular interest is the evidence suggesting that the transgene integrated into the genome of host cells. The authors went to great lengths to demonstrate integration using PCR, Southern blot, direct sequencing and fluorescence *in situ* hybridization. These data suggest the potential for designing viral vectors that combine the production properties, transduction efficiency and viral stability of Ad with the integrating properties of a retrovirus. Although, the efficacy of the hybrid Ad/MoMLV vector for transducing airway epithelia has yet to be investigated, the potential applications are intriguing.

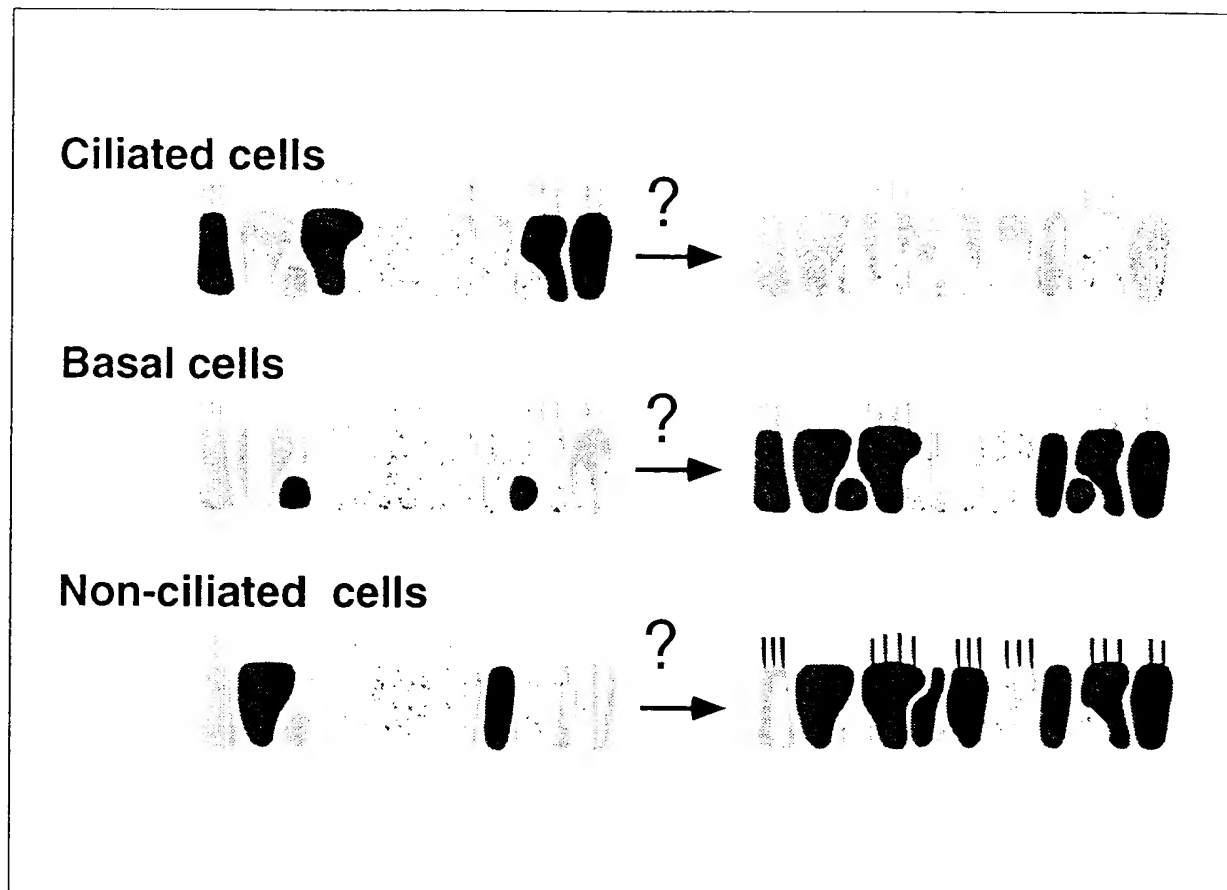
The possibility of combining attributes of two or more viruses to create viral hybrids or chimeras does not stop with retroviruses. For example, a great deal of effort has been applied to other combinations such as Ad and AAV [57,58]. It is likely that there will be continued development of hybrid systems combining the integrating potential of retroviruses with other vector systems to meet the challenges of the many gene therapy cell and tissue targets.

Targeting progenitor cells of the airway epithelium for persistent expression

In order to correct the pulmonary disease caused by CF using retroviral vectors it will be necessary to target a population of cells with progenitor capacity that can pass a correct copy of the *CFTR* gene on to their progeny. Only when this has been accomplished will persistent gene expression be possible with integrating vectors. Therefore, it is important to understand which cell types in the airways have progenitor capacity and design vector strategies to target those cells. With current pseudotyped retroviruses, a lavage approach may be required to facilitate access to receptors on airway progenitor cells, as some of these cell types reside below the mucosal surface, eg, basal cells, intermediate cells [59-62]. Alternatively, the identification of new envelopes with the capacity to target entry via the apical surface may also result in persistent gene expression.

Long-term *in vivo* studies will be needed to define the correct populations of cells to target to attain lasting correction of a genetic disease of the airway epithelium. Figure 4 suggests possible outcomes from such a study. For example, if a pseudotyped vector is used that can only transduce ciliated cells, and these cells have no progenitor capacity, gene expression will be transient. In contrast, transduction of non-ciliated surface cells or basal cells, cell populations believed to have progenitor capacity, may lead to clonal expansion and persistent transgene expression. Fortunately, the progress that is currently occurring in the field of retroviral vector development should make such comparative studies feasible.

Figure 4. Model of potential outcomes for transgene expression in the airway epithelium following gene transfer to specific cell types (ciliated, basal or non-ciliated).



Depending on the fate of the cell type that is first transduced with an integrating retroviral vector, lasting or transient gene expression may be attained. Transduced cells are indicated by dark gray.

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An In Vitro Model of Differentiated Human Airway Epithelia

Methods for Establishing Primary Cultures

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1. Introduction

1.1. The Airway Epithelium

The human airway epithelium forms a barrier between the external and internal environments, separating air from the interstitial space. However, it also serves many other functions. By active transepithelial transport of electrolytes, it controls the composition and quantity of the airway surface liquid covering the epithelium. It secretes numerous agents into the airway surface liquid, including IgA and antimicrobial factors; these form part of the defensive shield that protects the airways and lungs from infection. The activity of its cilia are key to mucociliary clearance. The epithelium participates in the inflammatory response when challenged with environmental factors or infectious agents. It responds to and produces a number of cytokines and other pro- and anti-inflammatory agents. To study and understand the complex and varied functions of human airway epithelia, investigators have developed cell culture models of the epithelium. Compared to in vivo studies, such models have the important advantage of flexibility, control of experimental conditions, and greater opportunities for intervention. They also allow the study of epithelial function in the absence of other cells and tissues such as macrophages, submucosal glands, fibroblasts, and cells of the immune system. Conversely, for some studies, the presence of nonepithelial cells and tissues would be advantageous.

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Importantly, in some cases, these other cell types can be added back to the epithelial culture system.

The surface epithelium lining the large human airways is pseudo-stratified; that is, all cells extend to the basement membrane, but not all cells extend to the luminal surface. Three of the most common cell types are the ciliated epithelial cells, goblet cells, and basal cells. For a description of cell types and morphology see Wheeler et al. (1). In the more distal airways, the epithelium is not as tall, it becomes columnar, and Clara cells become prominent. The cartilaginous airways also contain submucosal glands in the connective tissue layer beneath the epithelium.

This chapter describes a protocol to harvest and develop primary cultures of differentiated human airway epithelia from donor lungs and nasal polyps and turbinates. The epithelial cells are enzymatically dissociated from the airway tissue and seeded on permeable membrane supports. The cell culture with at least three different cell types develops into a pseudo-stratified epithelium with columnar cells supported on a collagen-treated membrane. This *in vitro* airway culture model has polarized epithelial cells with tight junctions and distinct apical and basolateral membranes. The culture is maintained with culture medium only on the basolateral surface, and the apical surface is exposed to air. The apical membrane surface develops microvilli and cilia and a covering of mucus. Thus, this *in vitro* model exhibits many properties comparable to the *in vivo* human airway epithelium.

1.2. Morphology of Primary Cultures of Differentiated Human Airway Epithelia

The differentiated human airway epithelial cultures we describe show morphologic properties similar to those of the airways *in vivo*. To examine the morphology, we have employed scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Fig. 1 shows an SEM image of the apical surface of a conventionally processed primary culture of human airway epithelia. This view shows the confluent arrangement of ciliated cells (C) and microvilli-covered goblet cells (G), which is characteristic of airway epithelia *in vivo*.

One limitation of conventional processing methods for electron microscopy (using aqueous-based fixatives and buffers) is that, for the most part, they remove the airway surface liquid (ASL) and mucus that cover the epithelial surface. Application of as little as 10 μ L of saline is sufficient to disrupt the ASL of the cultures. To examine better the overall morphology of the unaltered epithelial cultures, including the ASL, we have adopted a processing technique originally described by Thurston et al. and Sims et al. (2,3). This method uses osmium tetroxide dissolved in perfluorocarbon (PFC/OsO₄) as the fixative and avoids all aqueous reagents. The PFC is not miscible with water and

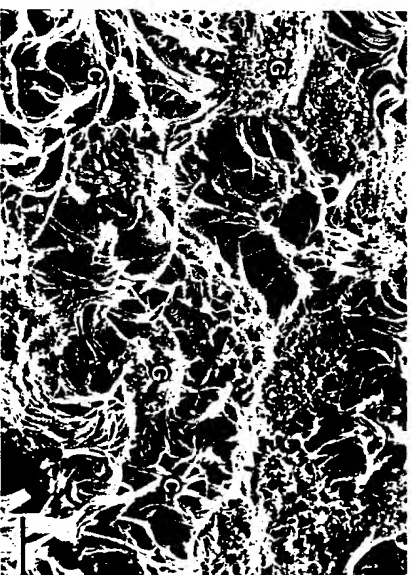


Fig. 1. Scanning electron micrograph of conventionally fixed and processed differentiated human airway epithelia grown on a semipermeable membrane filter. Visible are goblet cells (G) and ciliated cells (C). Scale bar equals 5 μ m.

does not disturb the ASL, leaving the OsO₄ free to diffuse into the ASL and the cells to stabilize the morphology. The cultures are then dehydrated with several changes of 100% ethanol and either transitioned to Eponate 12 epoxy resin and embedded for TEM sectioning or transitioned to hexamethyldisilazane and air-dried for SEM observation.

Fig. 2 shows a TEM cross-section of a PFC/OsO₄-processed airway epithelial culture. The morphology shows a classic pseudo-stratified epithelium, overlain by a layer of ASL as found in native human airway tissue. Identifiable cell types include basal cells (B), goblet cells (G), and ciliated cells (C). Cilia are visible within the ASL (arrowheads) some of which are in cross-section. Note the secretory granules contained within the goblet cells, with the cell on left having recently discharged much of its contents. Also visible are extensive interdigitations between the basal and adjacent cells. The 0.4- μ m pores in the filter are visible at the bottom of the image.

Fig. 3 shows an SEM image of a PFC/OsO₄-processed airway epithelial culture. This side-on view shows the edge of the epithelial culture, where the membrane was cut for removal. The apical portion of the epithelial cell layer has peeled back, exposing the ruffled surface of the basal cells (B), some of which remain adherent to the filter (F). The 0.4- μ m pores in the filter are also visible (arrowheads). Also note the mucus layer (M), which was not visible in Fig. 1. The mucus overlays the cilia (C) on the surface of the epithelium (E).



Fig. 2. Transmission electron micrograph of PFC/OsO₄-fixed human airway epithelia grown on a semipermeable membrane filter. Labeled structures include air, filter, ASL, cilia (arrowheads), goblet cells (G), basal cells (B), and ciliated cells (C). Scale bar equals 5 μ m.

1.3. Applications for Primary Cultures of Differentiated Airway Epithelia

Cultures of airway epithelia have been utilized for many applications. Here we mention several.

1.3.1. Measurement of Transepithelial Electrolyte Transport

Transepithelial transport can be evaluated by using radioisotope fluxes, or the epithelia can be mounted in Ussing chambers (Jim's Instruments, Iowa

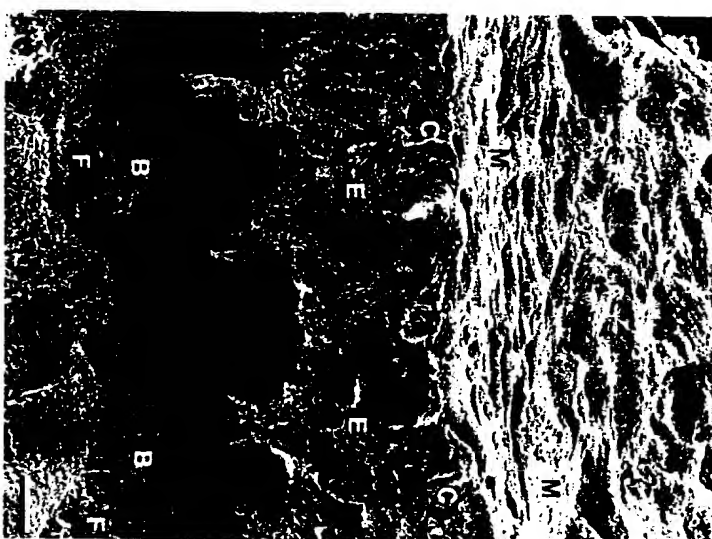


Fig. 3. Scanning electron micrograph of PFC/OsO₄-fixed differentiated human airway epithelia. View is from edge of filter where epithelium has separated from membrane filter. Labeled structures are filter (F), filter pores (arrowheads), basal cells (B), cilia (C), epithelia (E), and mucus layer (M). Scale bar equals 5 μ m.

City, IA) to measure electrogenic ion transport (4). Fig. 4 shows an example of the short-circuit current measured from differentiated airway epithelia. The tracing shows the amiloride-sensitive Na⁺ current and the cAMP-stimulated cystic fibrosis transmembrane conductance regulator (CFTR)-dependent Cl⁻ current.

1.3.2. Evaluation of Gene Transfer Using Viral And Non-Viral Vectors

Gene transfer offers the potential to be a new treatment for cystic fibrosis (CF) and other genetic and acquired diseases. However, when delivered from

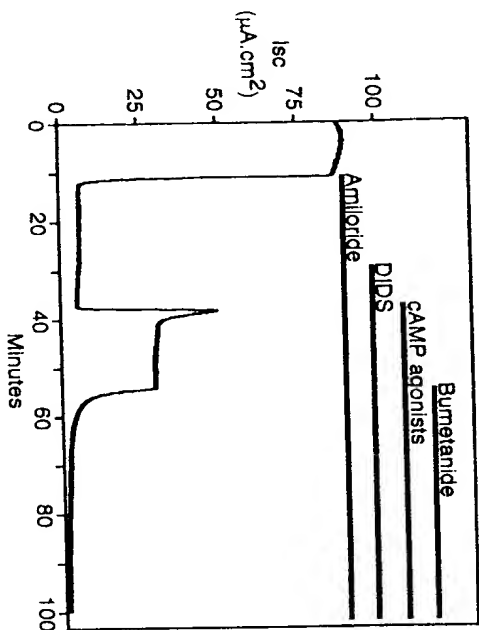


Fig. 4. Example of short-circuit current from differentiated primary culture of human airway epithelia derived from human bronchus. Tracing is from an epithelium mounted in a modified Ussing chamber, in which short-circuit current (I_{sc}) was continuously measured (558C-5 Voltage Clamp System, University of Iowa Biomedical Engineering, Iowa City, IA). Bars at top indicate sequential additions of the indicated compounds. Apical amiloride ($100 \mu M$) inhibits apical membrane Na^+ channels. Addition of 4,4'-disothiocyanato-stilbene-2,2'-disulfonic acid (DIDS; $100 \mu M$) to the apical surface inhibits Ca^{2+} -activated apical membrane Cl^- channels. The cAMP agonists forskolin ($10 \mu M$) and 3-isobutyl-2-methylxanthine (IBMX; $100 \mu M$) leads to phosphorylation and activation of apical membrane CFTR Cl^- channels. Basolateral addition of bumetanide ($100 \mu M$) inhibits the basolateral $Na^+-K^+-2Cl^-$ cotransporter. Epithelia were bathed in a symmetrical Ringers solution containing $135 mM NaCl$, $1.2 mM CaCl_2$, $1.2 mM MgCl_2$, $2.4 mM K_2HPO_4$, $0.6 mM KH_2PO_4$, $10 mM$ dextrose, and $5 mM$ HEPES (titrated to 7.2 with $10 N NaOH$). The Ussing chamber bath solution was continuously bubbled with $100\% O_2$.

the apical surface, most current vectors are very inefficient. Differentiated airway epithelia studied *in vitro* have proven to be an excellent model with which to investigate the underlying mechanisms for the inefficiency. For example, Fig. 5 shows that the receptor for adenovirus type 2 and 5 vectors, the coxsackie-adenovirus receptor (CAR), is located on the basolateral surface of the epithelium, where it is inaccessible to apically applied vector (5). The epithelia are also proving to be a very useful model for developing improved methods for gene transfer (6).

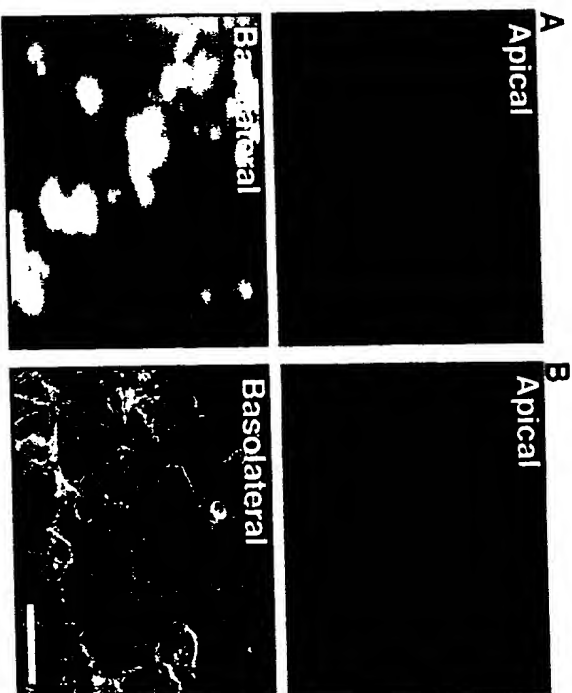


Fig. 5. Expression of the CAR and major histocompatibility complex (MHC) class I in well-differentiated human airway epithelia evaluated by immunocytochemistry. (A) Immunocytochemistry of unpermeabilized epithelia incubated with monoclonal anti-CAR Ab and an fluorescein isothiocyanate (FITC)-labeled secondary antibody applied to either the apical or basolateral side. Top panel shows absence of CAR staining on the apical membrane. Bottom panel shows a low intensity staining on most cells, with higher intensity staining of a small proportion of cells. (B) Unpermeabilized epithelia were incubated with an FITC-labeled monoclonal anti-MHC class I antibody applied to either the apical or basolateral side. Top panel shows absence of MHC class I staining on the apical membrane. Bottom panel shows high intensity staining on all cells. Bar represents $50 \mu m$. From ref. 5 with permission.

1.3.3. Models for Testing and Developing Novel Pharmaceuticals

Many pharmaceuticals are delivered via the airways, often as aerosols. The epithelial cultures can provide a system with which to investigate their effect on the epithelium (7). It can also be used to assess movement of agents across the epithelium to the smooth muscle, the interstitium, and ultimately the circulation. The converse is also true; the epithelium can be used to assess the movement of agents from the basolateral surface to the apical surface. An example is

the use of the polyclonal immunoglobulin receptor to shuttle agents from the submucosal to the mucosal surface (8).

1.3.4. Models for Disease Pathogenesis

Differentiated airway epithelia provide an excellent model for studying the pathogenesis of acquired and genetic diseases. An example comes from epithelia generated with cells obtained from people with CF. Studies have shown that the epithelia generate a defense system that can kill small numbers of bacteria, and that this system is defective in CF (9).

1.3.5. Models for the Interaction of Airway Epithelia with Environmental Agents

The airways are constantly being exposed to pollutants, toxic agents, and microorganisms. Differentiated airway epithelia provide an experimentally facile model system with which to evaluate such interactions. For example, they retain receptors and cytokine signaling networks that play key roles in inflammation.

1.4. Development of Primary Cultures of Human Airway Epithelia

Here we describe the methods and techniques required to harvest and culture human airway epithelia as an *in vitro* model of the airway. We focus on primary cultures, because in our experience, these have proven most reliable in yielding a functional and morphological phenotype that resembles the *in vivo* airway epithelium. We have not been able to consistently reproduce these features with cell lines originally derived from the airways. Even after very few passages, we find that the phenotype of the cells changes. Thus, at the present time in our hands, we find that a good model system requires the effort associated with primary cultures. Of note to the investigator studying airway epithelia, epithelia like those we describe here can be extremely useful when it is necessary to have a model that mimics airway epithelia. However, some experiments do not require such a model. When that is the case, there are other cells and approaches that may suffice with less work and expense.

The methods and procedures we describe here were adapted and modified from those originally reported in 1992 by M. Yamaya et al. (10). Thus, we owe them a debt of gratitude for their original work. The culture methods have been progressively modified to provide a high volume cell harvest that requires a reduced time investment and provides differentiated airway epithelia that remain viable for months. The adaptations have also enhanced the transepithelial transport properties of this culture method. We view the development of these cultured epithelia as both a science and an art. It is a science for standardization and purposes. It is an art because people vary in their technique, experience, and judgment, which produces adequate to excellent cultures. Moreover, the cell

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culture protocols we describe are a "work in progress". Future research will further improve the methods we describe here. We recommend that a person culturing these epithelia be willing to test a new media supplement, attachment substrate, or membrane support. When doing so, compare the culture results with the standard procedures.

2. Materials

1. Dulbecco's phosphate-buffered saline (PBS), Ca^{2+} - and Mg^{2+} -free (Sigma, cat. no. D5773). Store chilled stock at 4°C for rinsing surgical specimens. Store another stock at room temperature for rinsing Millicell membrane inserts.
2. Dulbecco's PBS with Ca^{2+} and Mg^{2+} (Sigma, cat. no. D5780). Keep chilled at 4°C for transporting surgical specimens.
3. Dissection kit:
 - a. Pair of medium size straight edge dissecting scissors.
 - b. Tissue forceps, which have jaws with fine teeth (Græfe Tissue Forceps, Bio-medical Research Instruments, Rockville, MD, cat. no. 30-1680).
 - c. Plasticware pan with a plastic liner, to keep the lung chilled on wet ice and to contain spills during dissection.
4. Minimal essential medium (MEM), Ca^{2+} - and Mg^{2+} -free dissociation solution. This solution is made from scratch and contains (per liter deionized water): 400 mg KCl, 6400 mg NaCl, 3700 mg NaHCO_3 , 125 mg $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 15 mg phenol red, 110 mg sodium pyruvate, 100,000 U penicillin, and 100,000 µg streptomycin. The pH of the solution is adjusted to 7.5 in room air with a few drops of concentrated HCl. Then it is filter-sterilized with a 0.22-µm membrane filter unit using vacuum. This solution can be stored for months at 4°C.
5. Dissociation enzyme solution. The two enzymes used in the dissociation solution are Pronase (Roche/Boehringer Mannheim, cat. no. 165921), which should be stored dry at 4°C and deoxyribonuclease 1 (Sigma, cat. no. DN-25), which should be stored dry at less than 0°C. For 100 mL, dissolve 140 mg pronase and 10 mg DNase in 100 mL MEM.
6. Fetal bovine serum (FBS) (Hyclone, cat. no. SH30070.03). It is not necessary to heat-inactivate the serum to remove complement. Moreover, it is preferable not to heat-inactivate the serum so as not to affect the potency of nutrient factors in the serum. Filter-sterilize the serum with a 0.22-µm filter unit to remove precipitate. Store at 4°C.
7. Dulbecco's modified eagle's medium (DMEM), high glucose (Life Technologies, cat. no. 12800082). Store at 4°C.
8. Ham's F-12 nutrient mixture (Life Technologies, cat. no. 21700091). Store at 4°C.
9. Ultrosor G supplement, a serum substitute (Bioprep SA, Cergy-Saint-Christophe, France, cat. nos. 259516 or 259515, USA distributor Crescent Chemical Co., Inc. Islandia, NY 11749). Store at 4°C. (A USDA import permit is required for USA import. The VS Form 16-3 application can be downloaded from web site: <http://www.aphis.usda.gov/N/CIE/>).

10. Airway medium 1: this medium is for cell seeding and the first culture day only. It is composed of a 1:1 ratio of DMEM and Hams F-12 supplemented with 5% FBS (not heat-inactivated) and 1% MEM nonessential amino acids solution (Life Technologies, cat. no. 11140-050).
11. Airway medium 2: this medium is for use after culture d 1 and then continuously. It consists of a 1:1 ratio of DMEM and Hams F-12 supplemented with 2% Ultrosor G. Freezing medium: 40% FBS, 50% airway medium 1, and 10% dimethyl sulfoxide (DMSO).
13. Culture antibiotics: we routinely add penicillin (100 U/mL) and streptomycin (100 µg/mL) (Life Technologies, cat. no. 15140-122), gentamycin (50 µg/mL) (Life Technologies, cat. no. 15750-060), fluconazole (2 µg/mL) (Diflucan; Pfizer), and amphotericin B (1.25 µg/mL) (Sigma, cat. no. A9528) to the two airway medias. The concentrations of antibiotics may be reduced, or the number of antibiotics reduced or eliminated after a few days in culture. For long-term maintenance of cultures, the combination of fluconazole and amphotericin B is especially effective in preventing or keeping in check yeast infection. For airway cultures from CF epithelia, we use additional antibiotics for the first 5 to 6 d of culture. The antibiotics are: 77 µg/mL ceftazidime (Fortaz; GlaxoWellcome), 2.5 µg/mL colistin (Coly-Mycin; Monarch Pharmaceuticals), and 12.5 µg/mL imipenem and cilastin (Primaxin; Merck). Culturing longer with the imipenem and cilastin will injure the epithelial cells.
14. Culture surface substrate for attachment and proliferation.
 - a. 60 µg/mL Collagen type VI from human placenta, acid soluble (Sigma, cat. no. C-7521). Store dry at less than 0°C until reconstitution. We routinely use this collagen as the preferred attachment factor for permeable membrane supports and plastic surfaces. We prescreen, in a small quantity, multiple available lots of the Sigma collagen because an occasional lot gives poor culture results on the membrane supports. We will then order a sizable quantity of a desired collagen lot and store it at -20°C for up to 18 mo.
 - b. Optional substrate: Vitrogen 100, bovine dermal collagen (Cohesion Technologies, Palo Alto, CA, cat. no. 0701). Prepare according to manufacturer's instructions. This substrate forms a gel surface support on the permeable membrane support.
15. Optional use: 100 ng/mL keratinocyte growth factor (R & D Systems, cat. no. 251KG-010).
16. Permeable membrane supports for culture of epithelia.
 - a. Millicell-PCF membrane inserts, 0.4 µm pore size, 12 mm diameter (Millicore cat. no. P1HP 01250). Millicore also makes the same PCF membrane insert with a 30 mm diameter (cat. no. P1HP 03050).
 - b. Optional use: A transparent membrane in a culture plate insert can be used as an alternative: Costar Transwell-Clear, 0.4 µm pore size, 6.5 mm diameter (Costar, cat. no. 3470). Costar makes the same clear Transwell membrane with a 12 mm diameter size (cat. no. 3460); our experience has been that different lots vary in terms of their ability to support successful cultures.

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17. Falcon Primaria tissue culture dish 100 × 20 mm (Becton Dickinson Labware, cat. no. 355803). Harvested cells are incubated on this plastic surface to remove fibroblasts prior to seeding cells on the permeable membrane supports.
18. Costar 24-well cell culture cluster dishes (Costar, cat. no. 3524). We prefer the Costar brand for uniform distribution of CO₂ to all Millicell inserts in the 24-well cluster dish.
19. Sterile 50- and 15-mL polypropylene conical tubes with screwcaps (Falcon, cat. nos. 352098 and 352097).
20. 150 × 75 mm Crystallizing dish (Fisher Scientific, cat. no. 08-762-9).
21. Hemacytometer counting chamber (Fisher Scientific, cat. no. 02-671-5).
22. Recommended optional equipment for measuring transepithelial resistance of airway epithelia grown on permeable membrane supports is a Millicell-ERS VoltOhmmeter (Millicore MERS, cat. no. 00001). Epithelial integrity can be quantitatively assessed with a portable instrument without compromising sterility or damaging the cells on the membrane.

3. Methods

3.1. Preparation and Dissection of Human Airway Tissue

Excellent viability of suitable human airway tissue is critical for the successful harvesting of epithelial cells and their subsequent culturing as polarized epithelia (see Note 1). We routinely use human donor lungs, but also use nasal polyps and turbinates as fresh specimens. Immediately after surgical removal, specimens should be chilled in either sterile cold physiologic saline, PBS with Ca²⁺ and Mg²⁺, or DMEM, and then transported in sealed containers on wet ice to the laboratory. Specimens should remain chilled during dissection.

1. For ideal aseptic and safety conditions, the dissection is best done in a laminar flow hood. The person should always wear sterile gloves. Handling human tissue and primary airway cultures may expose the user to airborne pathogens; suitable health protection measures should be followed.
2. Transfer all dissected tissue segments directly to 50- or 15-mL sterile polypropylene tubes for convenient processing and storage.
3. Rinse intact nasal polyps and turbinates in chilled sterile Ca²⁺- and Mg²⁺-free PBS (antibiotics are optional) in 50- or 15-mL sterile tubes to remove blood, mucus, and debris.
4. If necessary, the nasal tissue specimens can be picked apart from attached mucus and fibrous tissue in a sterile Petri dish. PBS rinsing may be required several times to remove blood clots.
5. Transfer the specimen to sterile 50- or 15-mL tubes containing the dissociation enzymes in chilled Ca²⁺- and Mg²⁺-free dissociation solution. The solution without divalent cations facilitates dissociation of cells.
6. Airway tissue from the lung includes the attached trachea, the main stem bronchi, and the lobar bronchi, which divide repeatedly in the pulmonary lobe. We dissect bronchial segments down to the third segmentation (see Note 2).

7. Intact segments of the trachea and bronchi (containing the epithelium, smooth muscle, and cartilage) should be separated from extraneous connective tissue using scissors and cut into longitudinal 2–4 cm sections. Segments of smaller diameter bronchi are cut longitudinally to maximize lumen exposure.
8. Place the segments in polypropylene tubes with cold DMEM.
9. Rinse with Ca^{2+} - and Mg^{2+} -free PBS multiple times to remove blood, mucus, and purulent material from the lumen.
10. Airway segments can be immersed in cold Ca^{2+} - and Mg^{2+} -free PBS for 1 h to help remove blood clots. The segments can then be transferred to new 50- or 15-mL polypropylene tubes containing the chilled dissociation solution.
11. Segregate segments in different tubes according to airway region and lobe, because of possible variation in viability of the epithelium in the different branches of the airway.
12. It is important to prevent warm and cold temperature changes of airway specimens during processing to prevent epithelial degradation.

3.2. Dissociation and Harvesting

1. On the day of use, prepare the dissociation solution by dissolving the two enzymes, pronase and deoxyribonuclease, in the previously prepared stock of Ca^{2+} - and Mg^{2+} -free MEM (see Subheading 2, step 4). In the MEM solution, dissolve 1.40 mg pronase and 10 mg DNase per 100 mL. Stir to dissolve enzymes and then saturate the solution with 5% CO_2 in either air or 95% O_2 to provide an optimal pH. We use a line from the gas cylinder to bubble the solution for several minutes in a beaker covered with parafilm. Then sterilize the solution with a 0.22- μm filter and transfer to new sterile 50- or 15-mL polypropylene tubes.
2. Store the dissociated airway specimens in the tubes with the enzyme dissociation solution at 4°C for 24–96 h. Occasionally invert tubes during dissociation to agitate and break apart cell clumps.
3. Nasal specimens can be dissociated for 24–48 h. Trachea and bronchus tissue require a minimum of 40 h to a maximum 96 h. The time for dissociation depends on the desired degree of separation into single cell vs cell clumps (see Note 3).
4. Aliquots can be examined with a microscope on a glass slide to assess the degree of clumping over time.
5. To end dissociation, add 10% (v/v) FBS to the dissociation solution. Invert the tube(s) several times to agitate the cell suspension.
6. Transfer the harvested cell suspension to new polypropylene tubes with a pipet.
7. Rinse tissue segments with chilled sterile DMEM or Hams F-12 to collect additional cells.
8. Centrifuge the cell suspension at 120g for 5 min.
9. Resuspend the cell pellet in airway media 1 (5% fetal calf serum [FCS]) with antibiotics at 37°C . Gently break apart the cell pellet by pipetting with a sterile short stem Pasteur pipet. Be careful not to pipet too vigorously. The harvested cell suspension will consist of single cell and multiple-cell clumps.
10. Transfer the cell suspension by pipet to Primaria tissue culture dishes. A cell strainer is optional to filter large clumps and tissue debris (see Note 4).

11. Incubate the suspension in a CO_2 incubator at 37°C for a minimum of 1 h or longer. This incubation allows fibroblasts within the cell suspension to attach to the plastic surface. Airway epithelial cells will not attach to the plastic surface without collagen pretreatment. We have found that the Falcon Primaria tissue culture plastic works best for enhanced fibroblast cell attachment. The numbers of fibroblasts in the airway cell suspension varies with different lung specimens. Occasionally, a specimen yields densely attached fibroblasts so that a second incubation with a new Primaria dish is required. If too many fibroblasts are present in the seeded suspension, and they attach with the epithelial cells to the permeable membrane support, the fibroblasts will prevent the cells from forming an intact epithelium with a good transepithelial resistance, because there will be intermixed colonies of fibroblasts that do not form tight junctions.
12. Total epithelial cell yield will vary depending upon tissue viability and size. An adult lung with trachea and 2 to 3 generations of bronchi will yield in the range of 5×10^7 to 2×10^8 epithelial cells.

3.3. Substrate Preparation

- Primary human airway epithelial cells will not attach to the Millicell membranes without surface pretreatment. We coat the top of the Millicore PCF membrane insert with a solution of human placental collagen (60 $\mu\text{g}/\text{mL}$) for a minimum of 18 h. The collagen solution can be stored on the membrane supports (12 mm diameter) for several days in 24-well cluster dishes at room temperature.
1. Use a ratio of 30 mg collagen with 50 mL deionized water and 100 μL glacial acetic acid.
 2. Cover the holding beaker with parafilm and stir moderately at about 37°C until collagen strands are dissolved. We use a crystallizing dish containing warm (up to 37°C , changed approx every 5 min) tap water as a make-shift water bath and place it on a magnetic stirrer. The time required for the collagen to dissolve varies significantly. Fifteen to twenty minutes is usually sufficient.
 3. Dilute the filtered collagen stock 1:10 with deionized sterile water. This diluted collagen stock (60 $\mu\text{g}/\text{mL}$) is the working solution for coating plastic and membrane surfaces. The working collagen stock can be stored in a glass bottle at 4°C for several weeks.
 4. Filter-sterilize with a 0.2- μm membrane. If the filter membrane quickly plugs, the collagen strands have not fully dissolved.
 5. Collagen-coat the surface of the membrane for a minimum of 18 h at room temperature. The collagen improves cell attachment efficiency and proliferation. Shorter times may work in an emergency. The liquid collagen can remain at room temperature on the membrane for several days.
 6. On the day of cell seeding, remove the liquid collagen from surface and air-dry the membrane surface.
 7. Rinse at least twice with sterile PBS or DMEM on both sides of membrane support. It is important to remove all trace of the liquid collagen. Residual liquid collagen can be toxic to cells.

8. An alternative substrate is a Vitrogen gel, which is prepared on top of the membrane before seeding. This substrate has advantages and disadvantages. One advantage is that the columnar cells appear taller. One disadvantage is that it is more difficult to work with and maintain stable airway cultures because the gel is fragile (see Note 5).

3.4. Seeding Cells on the Membrane

1. After collecting the nonattached cell suspension from the incubation dish in airway medium 1 (5% FCS), the cells are counted to determine cell density.
2. We recommend manual counting with a hemacytometer counting chamber and viewing the cell suspension under a microscope. By looking under higher magnification (400 \times) at an aliquot from the cell suspension, one can make judgments about the viability and homogeneity of the airway epithelial cells. The hemacytometer count will underestimate the number of cells if there are sizable cell clumps present in suspension; the cover glass prevents large clumps uniformly covering the grid area. A complementary approach is to also use an aliquot thinly smeared onto a glass slide; this allows viewing of the representative degree of clumping in the cell suspension. We include a "fudge factor" for larger clumps when calculating cell suspension density. The greater the degree of clumping, the lower the calculated seeding volume derived from the hemacytometer count. The cell suspension may be heterogeneous, ranging from single cells to multiple-cell clumps. There may occasionally be other nonepithelial cells in the suspension, including red blood cells.
3. Follow the instructions of the hemacytometer manufacturer. Count at 400 \times magnification only the epithelial cells that have a distinctly dark cell membrane appearance and that are noncolumnar in shape. Cells that are pale in appearance, cells with a broken cell membrane, or cells that appear empty are not counted. Nonmotile ciliated cells are also not counted. Smaller cells that have a halo appearance are red blood cells and are not counted. Estimate the number of cells per clump and include that in the total cell count.
4. Our experience with using the Trypan blue exclusion assay with the primary cultures of human airway epithelial cells is that it is inadequate for assaying nonviable cells. Except when counting viable cells thawed from cryostock, we recommend not depending upon the dye assay for an accurate determination of cell viability.
5. It is best to try a range of seeding densities based upon your manual count method until you are comfortable with the accuracy of your counts and can correlate the counts with the later successful confluence of the airway culture on the membrane support. The seeding range that we use is 2.5×10^5 to 5×10^5 epithelial cells/cm² on the Millicell insert. Cell harvest suspensions that have more clumps than "average" can be seeded at the lower density range. There is a higher efficiency of attachment with cell clumps than single cells, especially with ciliated cells (see Note 3). Confidence with the appropriate seeding density of primary epithelial cells requires trial-and-error experience. The presence of red blood cells in the cell suspension will not interfere with epithelial cell attachment.

6. The cell suspension seeded onto the top of the permeable membrane insert (12 mm diameter) should be of sufficient volume, 100–400 μ L, to insure a uniform distribution of cells settling upon the membrane surface. The volume of medium under the membrane insert should be sufficient (250–500 μ L) to immerse the membrane bottom without floating the insert.
7. After seeding, make sure that the membrane supports are level to insure uniform cell attachment during the first 12 h. We use 24-well cluster dishes to maintain the 12-mm membrane inserts. The 6-well cluster dish can be used with the larger 24- to 30-mm inserts.
8. Leave the cluster dishes with the seeded membrane supports undisturbed for a minimum 18–24 h in a CO₂ incubator at 37°C and 8 to 9% CO₂ (see Note 6). The higher CO₂ increases successful achievement of confluence, especially with CF airway cultures.
9. The day after seeding, change the airway medium 1 (5% FCS) on both sides of the membrane to airway medium 2 (2% Ultrosor G).
10. Remove the top medium with vacuum suction and rinse the top of the membrane surface once with airway medium 2 to remove unattached cells.
11. Then remove the medium from the top of the membrane surface (air interfacing) so that medium is present only on the bottom of the membrane insert. When cells grow at the air interface, they form a confluent sheet with tight junctions and no visible fluid on top. The air interface allows the confluent sheet to better differentiate as a barrier separating air on top from the liquid media immersing the bottom. This air interface condition is comparable to the air-covered surface of the airway epithelium *in vivo*.
12. Remove any liquid from the top surface once daily until the membrane culture remains visibly dry on top; which usually occurs 3–6 d after seeding in 8 to 9% CO₂. Media seepage will occur through the membrane and the nonconfluent cell cultures for several days until the cells replicate to confluence.
13. Maintain the airway medium 2 on the bottom side of the membrane insert for the entire culture duration to prevent the culture from drying and the cells dying.
14. After the cells achieve confluence, the polarized sheet of epithelial cells will regulate the minimal fluid level and content on top. The amount of liquid is so small that it is not visible to the eye.
15. For cryopreservation of primary airway epithelial cells, resuspend cells in freezing medium. Cryovials are frozen at –70°C for 4–24 h and then transferred to liquid nitrogen for long-term storage.
16. At the time of later use, thaw the cryovial in a 37°C water bath and rinse with airway medium 1.
17. Centrifuge and resuspend the cell pellet in airway medium 1. Up to 70% cell viability is possible.

3.5. Routine Culturing and Long-Term Maintenance

Two cell culture conditions facilitate the long-term viability of polarized human airway epithelial cells on membrane supports. The combination of the airway medium 2, which contains 2% Ultrosor G, and the apical air interface

allow the epithelium to remain viable for months. Our data suggest that the epithelium probably does not continue to differentiate past approx d 14 in culture. Long-term culture maintenance is most economical and allows for time flexibility in use of the epithelium in experiments. We have maintained viable airway epithelia on membrane supports for up to 15 mo. During this time, the epithelia maintain an air interface and a transepithelial resistance. Such long-term preservation can be especially valuable for studies designed to evaluate the persistence of transgene expression following viral or nonviral vector-mediated gene transfer.

1. After the first 4–6 d of culture and once the air interface can be maintained by the cells, keep the cultures in a 5% CO₂ humidified atmosphere at 37°C for the remainder of their time in culture.
2. Beginning the day after seeding, any liquid on the top surface must be removed daily until the epithelium becomes confluent and can maintain the air interface. Long-term viability requires an air interface at the apical membrane.
3. We estimate that between 50–70% of the seeded airway cells will attach to the collagen-coated membrane within 12 h after seeding. Cell replication continues until 7–9 days after seeding. By the ninth day there will be on average 650,000–750,000 airway epithelial cells on a 0.6-cm² membrane surface. Cell numbers will then remain relatively constant unless a growth factor such as the keratinocyte growth factor (KGF) is introduced into the basolateral cell culture medium to initiate cell replication (11).
4. The rate of medium acidification by the cell culture varies depending upon the age of the culture. Younger cultures will require more frequent medium changes every 2 to 3 d. Older cultures require a medium change every 5–7 d. Change the medium whenever it becomes too acidic, as indicated by a yellow appearance. It is critical that the membrane surface not be touched by a pipet tip when changing media to prevent damage to the epithelium and a reduction in transepithelial resistance.
5. The greatest challenge to maintaining cultures for weeks and months will be preventing infection by opportunistic pathogens, especially yeast. We have found the combination of two fungicides, fluconazole and amphotericin B, are especially effective in preventing infection or limiting exponential growth after initial infection. Decreased antibiotic concentrations or complete elimination of antibiotics is also feasible for extended periods of time if required by an experimental protocol. We pre-incubate culture medium in filter-top flasks in the CO₂ incubator prior to use in order to screen for any contamination of media stocks. Especially for long-term culturing of up to a year, we will "spread the risk" of loss due to incubator failure or mechanical problems by using two or more CO₂ incubators for different trays of epithelia from the same specimen. Before every change of media, we inspect each multiwell tray of epithelia for signs of media discoloration and turbidity that would indicate contamination. We also alternate days for media changes of different airway cultures to reduce the chances of cross-contamination from one contaminated culture.

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6. Airway epithelia are maintained long-term with airway medium 2 present only on the bottom surface. If the experimental protocol requires supplement depletion, the epithelia can remain viable without the Ultrosor G supplement for several days in the 50/50 DMEM and Ham's F-12 medium.
7. Additional medium supplements can be added for special needs. For example, to reinitiate cell replication following confluence, we use keratinocyte growth factor for 24–48 h during days 8–10 post-seeding.

3.6. Evaluation of Airway Epithelia

1. The evaluation of the cultures is critically important to determine that they have the phenotype associated with airway epithelia. During the process of culturing, they must be continuously examined in several ways.
2. For example, check to see if the apical surface appears dry. Routinely check the transepithelial electrical resistance (R_t) and the morphology on every set of cultures.
3. We also study several epithelial cultures from every airway specimen in Ussing chambers to measure R_t, transepithelial voltage, the amiloride-sensitive Na⁺ current, and the CFTR-dependent Cl⁻ current.

3.6.1 Transepithelial Resistance.

Here, we briefly describe the measurement of R_t and evaluation of morphology. Measurement of transepithelial electrical properties in Ussing chambers will not be described. To learn more about those procedures, the reader may consult other references (12,13).

1. R_t can be conveniently monitored using a portable ohmmeter. We use the VoltOhmmeter attached to dual "chopstick" electrodes.
2. Each of the two electrode stems contains at their tip a Ag/AgCl electrode for measuring voltage and a concentric spiral of silver wire for passing current across the epithelium.
3. Presterilize the electrode tips in 70% alcohol before use to maintain cell culture sterility.
4. To measure electrical properties, place 300–400 µL of airway media 2 on the apical surface. It is removed after measurement.
5. Using a culture membrane insert such as the Millipore PCF, place the shorter electrode tip into medium on top of the apical surface, and place the longer electrode tip into the external bathing medium.
6. Current can then be passed across the epithelium to measure R_t. R_t values higher than the background fluid resistance indicate a confluent airway epithelium with tight junctions.
7. In our experience, we have found that R_t measured in this way is consistently higher than that obtained with the more accurate measurements obtained when the epithelia are studied in Ussing chambers. This difference is probably due, at least in part, to the fact that the distribution of current across the epithelium is more uniform in Ussing chambers. In contrast, with the chopstick electrodes, the

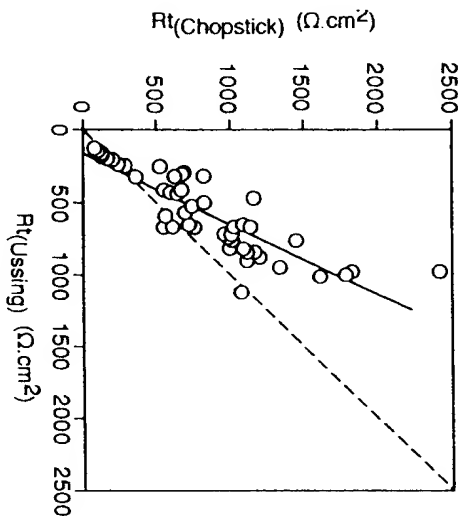


Fig. 6. Comparison of measurements of transepithelial electrical resistance in primary cultures of human airway epithelia using a chopstick ohmmeter, $R_t(\text{Chopstick})$, and measured in Ussing chamber, $R_t(\text{Ussing})$. Both measurements were made on the same epithelia. Measurements made with the chopstick ohmmeter consistently overestimate the true R_t . The dashed line is the line of identity, and the solid line is the linear fit to the data.

distribution of current flow may be influenced by spatial constraints independent of the permeability of the epithelium. We have compared R_t values measured by the Millipore ERS Volt ohmmeter with R_t values measured by voltage-clamp in Ussing chambers. The data are shown in Fig. 6. In both cases, the electrical resistance of the fluid and the filter were subtracted, and the measurements were corrected for the area of the epithelium.

The relationship is described by the formula:

$$R_t(\text{Ussing chamber}) = (0.492 \times R_t(\text{chopstick})) + 158$$

When area and fluid resistance are not corrected for measurements taken with the chopstick electrodes, the relationship is described by the formula:

$$R_t(\text{Ussing chamber}) = (0.295 \times R_t(\text{chopstick})) + 107$$

3.6.2. Morphology

1. On every set of cultures you should use SEM to evaluate the morphology of the apical surface. As the epithelia mature and differentiate, apical surface morphol-

- ogy changes. Fig. 7 shows SEM photomicrographs taken at increasing durations after seeding epithelial cells from a human bronchial specimen (14).
2. Three days after seeding, the cells appear confluent (Fig. 7A,B). The cells vary in size and most appear to have short microvilli. However, there are very few ciliated cells. Six days after seeding, most of the cells appear to have immature cilia (Fig. 7C,D). On d 10 (Fig. 7E,F) and 14 (Fig. 7G,H) after seeding, the surface of the monolayers is covered completely by cilia, making it difficult to identify individual cells. In some areas, there is also amorphous material mixed in with the cilia.
3. In our experience, by 14 d in culture, the epithelium has fully differentiated and is ready for use in experiments. Airway specimens can vary greatly in the percentage of epithelia cells that develop cilia in culture. This may reflect, in part, the history of the human donor. For example, chronic exposure to cigarette smoke or other insults or disease, which damages the airway epithelium *in vivo*, may influence *in vitro* results. Increasing the degree of clumping of ciliated cells by shortening the dissociation time may increase the proportion of ciliated cells that attach to the membrane within the first 12 h.

4. Notes

1. Development of successful primary airway epithelial cultures depends on the viability of the original tissue. The harvested cells need to be viable and in sufficient quantity to ensure an equitable distribution of viable cells seeded on the permeable membrane supports. This should then later produce confluent epithelia with intact tight junctions. With these requirements, it can be problematic to obtain viable human airway specimens in a quantity to yield a significant cell harvest. We have obtained human airway nasal tissue and lung from several sources. They include nasal polyps and turbinate following endoscopic polypectomy or endoscopic sinus surgery. The polyps need to be harvested intact, using a scissors or sharp manual dissection, and not minced by a mechanical microdebrider, which is increasingly popular for polypectomy. We have obtained poor viability of epithelial cells harvested from minced specimens.

We have also tried to use tracheas and lung specimens obtained postmortem. This source is problematic due to the time between death and harvesting the tissue and the greater risk for bacterial and yeast contamination. Rinsing airway epithelium with water at the postmortem surgery site is to be avoided. Our experience has been that we had occasional success with cells obtained from the trachea, but the viability of cells of lower airway was poor.

Our best success in obtaining high quality cultures has come when we used viable trachea and bronchus segments from human donor lungs. These lungs have been prepared and harvested aseptically according to transplant standards, but have then been rejected for human transplantation. The lungs are immersed in sterile saline and sealed in sterile bags, which are then chilled by immersion in wet ice. Lung specimens usually arrive in the laboratory within 6–18 h following removal. These lungs come from donors of all ages and medical histories. The donor lungs have been rejected for human transplantation due to donor age older

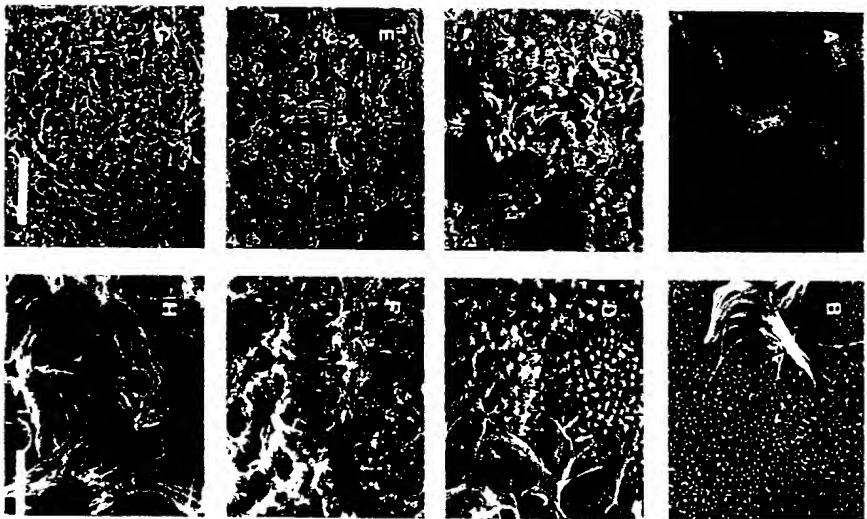


Fig. 7. Scanning electron photomicrographs of primary cultures of human airway epithelia grown at the air-liquid interface. Photomicrographs of monolayers are shown at the following times after seeding: 3 d (A,B), 6 d (C,D), 10 d (E,F), and 14 d (G,H). In panels A, C, E, and G, the scale bar indicates 37.5 μ m, and in panels B, D, F, and H, the scale bar indicates 5 μ m. On d 3, sporadic ciliated cells can be observed surrounded by undifferentiated cells with short microvilli. By d 6, most of the cells have cilia (thin arrows in panel D) or immature cilia (thick arrows). By d 10 and 14, most of the cells are ciliated. From ref. 14 with permission.

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than 60, presence of sepsis, chronic smoking exposure, or lung damage due to bruising and bleeding. We dissect and segregate tissue segments according to the airway region and lobe because of possible variation in epithelial viability. Airway epithelium from CF lungs are especially variable in viability from area to area due to chronic inflammation and infection.

A commercial source for cryofrozen primary or first passage human airway cells is Clonetics (Walkersville, MD). This source is economical when the cells are serially passaged to expand cell numbers. However, based upon comparative studies using primary and passaged airway cells, our laboratory prefers the morphology and functional properties of the primary cells, because they more closely resemble those of the airway epithelium *in vivo*.

1. The lobar bronchi divide repeatedly with each branch, becoming progressively smaller in diameter. Our experience has been that the smaller airway segments are less likely to yield viable cells, and the yield will be smaller. In considering the trade-offs of time and cost vs viable cell yield, we routinely end dissection after the third bronchial branching. However, special needs and protocols may require harvesting cells from smaller bronchi.
2. Dissociation time can vary depending upon requirements. The longer the dissociation time, the finer the single-cell dissociation. Aliquots removed at staggered times from the dissociation tubes and microscopically examined on a glass slide can be used to assess the degree of dissociation. It is important to determine by experience what will work best for you. Small clumps of cells (approx 4–10 cells/clump) have a higher efficiency of attachment than single cells and provide cluster seeds for cell division. We usually harvest cells about 60–72 h after starting the dissociation.
3. For certain protocols, it may be desirable to remove or segregate large-size clumps of airway cells from the cell harvest suspension. A sterile 100- μ m nylon cell strainer (Falcon, cat. no. 2360) can be used for this purpose.
4. Vitrogen gel substrate. According to the manufacturer's insert, mix in an 8:1:1 ratio of the cold Vitrogen 100, sterile cold 10x DMEM, and cold 0.1 N NaOH. Using pH indicator strip paper, adjust the solution pH to 7.4 with 0.1 N HCl and 0.1 N NaOH. The collagen solution is then aliquoted onto the top surface of the membrane support (150–200 μ L/0.6 cm²). Then warm the coated membrane surface to 37°C (not in CO₂), for a minimum of 1 h to gelate the collagen. Then use the gel for cell seeding within 3 h. Rinse the collagen surface with medium prior to seeding cells. Do not use vacuum suction on the collagen gel surface. Use a handheld suction pipet bulb to avoid damage to the collagen gel. This gel substrate is fragile during culturing. Our experience has been that there is a steady attrition rate. Therefore, we always cultured more than we needed for a particular experiment.
5. For the first 4–6 d, we routinely culture the airway cells on the membrane in a high CO₂ atmosphere (8 to 9%) until the culture can maintain an air interface. Then the cultures are transferred to a 5% CO₂ atmosphere for long-term maintenance. Not all airway specimens require this enhanced CO₂ condition. But there

is a critical time window during the first several days of culture for the cells to grow to confluence and form acceptable transepithelial resistances. If the cells have not become confluent by d 6 after seeding, the rate of later confluence success is low. We have observed a higher success rate using an initially higher CO₂ atmosphere for our airway cultures.

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